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Recombinant DNA—how public?

A PROBLEM increasingly facing scientists is the pressure to become involved in public discussion of the assessment of risk. Although initial fears about potential health hazards of recombinant DNA research have proved to be exaggerated, the public discussion has required scientists to take a stand on the risks involved—just as public policy decision in other fields, such as the control of chemical carcinogens, has needed assessments that can be backed up by scientific data. In both instances there are two, related, requirements: the first is a reasonable interpretation of available data, and the second is that the public should have confidence in this interpretation.

Much thinking has, of course, been taking place on both sides of the Atlantic on how best to relate the scientific to the policy aspects of risk assessment (the need for which is now generally accepted even by those who continue to dispute whether such assessment can be reduced to cost/benefit analysis). Some have argued strongly for a clear separation of the scientific and regulatory aspects. The US National Academy of Sciences, for example, in a report on food additives published in Washington last month, suggested that it should be a scientific task to divide potentially harmful food additives into three categories of risk, and then a regulatory task to decide how substances in each category should be dealt with. And staff members of the President's Science Adviser's office have suggested a broadly similar, interagency approach to all areas of regulation of carcinogenic substances (page 388).

Such an approach has obvious attractions but in practice it is often difficult to draw the dividing line between the scientific and the political judgement. Frequently there is a lack of adequate scientific data to reach a firm conclusion on the subject under debate—the effects of low level radiation, or even the health hazards of recombinant DNA research, are two obvious examples. In such cases, a variety of interpretations can be compatible with such data as are available. And the choice between different interpretations is one that expresses social or political interests (those who wish to pursue a certain line of research, whether the nuclear industry or DNA research workers, will tend to favour the interpretations that minimise the hazards—their critics will tend to take the opposite view).

One should not be surprised at this situation, merely aware of the bias that will inevitably creep in. But one

thing that is certain is that to achieve a reasonable and acceptable policy position, the public debate on such risks must be as open as possible.

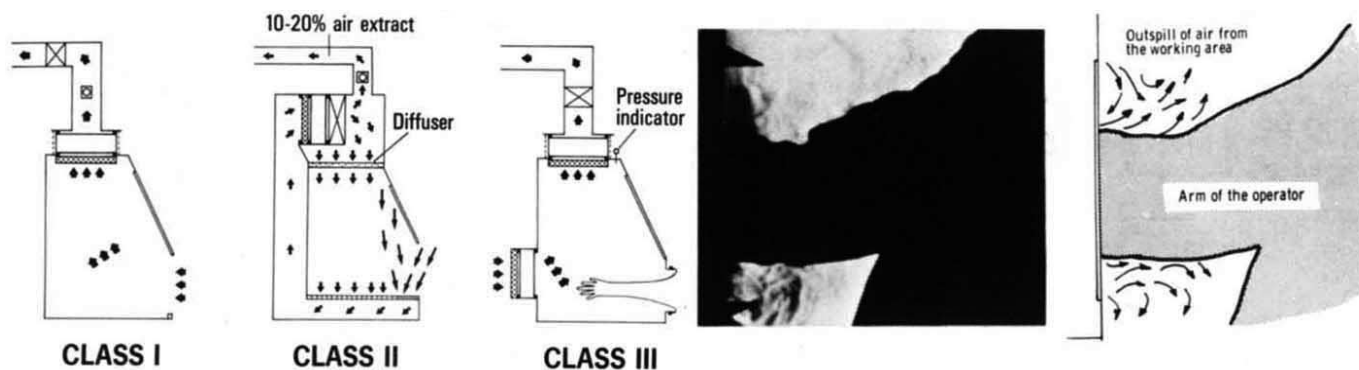
What then are we to make of the restrictive notice from the organising committee of the forthcoming international scientific meeting on recombinant DNA at Wye College, Kent, which puts the whole meeting 'off the record'? The purpose of the meeting, supported by ICSU and the Royal Society, is 'to review the current status of DNA research and technology, and to discuss matters of concern such as support for the research, its regulation through guidelines and legislation, and the eventual practical application of the technology'; widespread participation is called for. And yet participants have now been told that for 'free and frank discussion usual to scientific meetings' the proceedings will be strictly off the record, and that everyone should respect the 'established convention of private communication between scientists, and not contribute to any reviews for publication other than . . . the official proceedings'. Originally the press was to be excluded entirely, but now three science writers will attend (from *Nature* and *New Scientist*) provided they refrain from recording the meeting, or writing reports for general publication directly from the proceedings.

Of course scientists working in this field may at times have felt hard done by the media. And of course it is open to journalists to get the material by the alternative route of dogged pursuit of participants for on-the-record comment. But the committee has already clearly set the tone—the discussions are too sensitive to be widely disseminated, except in an official version. Others may conclude—perhaps quite wrongly—that there are other things to hide.

Moreover, it is never possible for all those who wish to attend to find the time and money to do so. Sober reporting of the proceedings fulfils an important function of allowing the whole community, not just those able to get to a particular place, to gain at least some impression of what went on.

Science, we are continually reminded, is public knowledge, freely available. Anyone lecturing to a large audience, accumulated by advertisement as well as by invitation, is participating in this tradition, not conforming to an 'established convention of private communication'. The committee should think again. □





‘Safety’ cabinets are not safe, says UK study

Some of the most popular makes of safety cabinet used for biological research in the UK have containment factors 1000 times lower than the new British Standard, writes **Judy Redfearn**.

WORK sponsored by the UK Medical Research Council has shown that the vast majority of ‘safety’ cabinets currently being used for microbiological work in hospitals, universities, and research establishments throughout the UK offer so little protection to those working with them that the term safety cabinet is a misnomer. An important and positive outcome of this work, however, has been the preparation of the first British Standard (BS 5726) for safety cabinets, which is due for publication “any time now”. The standard will define three classes of cabinet (see figure) and recommend minimum containment levels for each.

The cabinets which are causing most concern have a linear downflow of air at the opening. Of the dozen or so makes of this type (class II) currently available in the UK, only two, the UK-manufactured Howarth and the Italian Gelaire, have been found to come up to the new BS recommendation for containment.

Some popular makes of cabinet to have containment factors up to 1,000 times less than the recommended minimum. According to Dr R. P. Clark of the Clinical Research Centre, Harrow who has done much of the work for the MRC, in some cases and for some uses a microbiological worker could be at greater risk when using a cabinet than when working on the open bench—because the air flow created by the cabinet could cause infectious material to be blown directly at his or her face.

Class II cabinets are very popular for work where total containment is not thought necessary, because they are had been thought to offer some protection both to the worker (from inhaling infectious particles) and to the work (from contamination from the environment). The latter can be important during work with tissue cultures.

The cabinets operate by drawing air through the front opening and down through a grille in the floor. The air

then moves behind the cabinet and through high efficiency filters before re-entering the cabinet through the roof. During normal operation, about 80-90% of the air is recirculated back into the cabinet and the remaining 10-20% is filtered and discharged to the atmosphere. Drs Clark and B. J. Mullan used Schlieren photography to look at the air flow in and around cabinets running with and without an operator. They also examined the effect on the air flow of a person walking past the front of the cabinet or of a door opening and closing near it, of talking or coughing towards the cabinet and of a lighted Bunsen burner inside it. Behaviour of the air flow was also examined after switching the cabinet off.

For all the cabinets tested they found that in the first 3s after switch-on a “large rolling eddy” of air spilled out of the front opening. The flow pattern then settled down and a substantial bulge of air began to circulate regularly outside the cabinet. Some air was convected away from the bulge to mingle generally with the air of the room.

The effect of an operator working with the cabinet was to cause turbulence in the air bulge outside. When a smoke pellet was ignited in the cabinet, smoke was clearly seen emerging through the front opening above the operator’s hands and moving towards the face. The effect of someone coughing or speaking towards the cabinet or walking past the front of it, or of a door opening and closing near it, was to disturb the air bulge and cause it to mingle with the air in the room. After the air flow through the cabinet was switched off, the warm air inside moved upwards and outwards from the front opening and continued to do so for 10-20 min.

Drs Clark and Mullan have tried to quantify these disturbing observations by measuring the containment of various makes of cabinet. They created an aerosol of potassium iodide solution inside each one and measured the

number of droplets contained per unit volume of air just outside the front opening. They then performed the same experiment on the open bench and calculated the ratio of the measurement made on the open bench to that made with the cabinet.

This work is the basis of the new British standard. The standard will recommend that Class II cabinets (with a dummy arm inserted in the front opening) offer 10^3 times more protection to the operator than the open bench.

When tests first started at the Clinical Research Centre no UK-manufactured cabinets came up to this specification and some had containment factors of only 10^2 . In the light of this work, however, one UK manufacturer has designed a new cabinet, the Howarth, which meets the BS specification. The cost of this model compares well, according to Dr Clark, with the cost of other less adequate cabinets. However it is disturbing, says Dr Clark, that some manufacturers are now rushing to sell off their old stock. He is aware of two firms taking orders in the last two weeks and threatening cancellation charges if the orders are withdrawn when the standard is announced.

But if—against this trend—the publication of BS 5726 goes hand in hand with a willingness of other manufacturers to substitute better designs, then the biologists will at least have started to tackle their major safety problem. Fume cupboards for chemical work, however, are likely to remain without a British Standard for some years yet. □

Figure: cabinets in Class I (left) are designed to protect the worker but not the work. Those in Class II (second left) are for protecting both the worker and the work—as are those in Class III (centre). The latter are for work with highly infectious or pathogenic organisms where total containment is needed. Right: what happens when an operator disturbs the air curtain in Class II cabinet.

US drug companies push for changes in recombinant DNA guidelines

David Dickson reports that fears of industrial espionage are making drug companies reluctant to comply with rules covering genetic engineering research

US pharmaceutical manufacturers are challenging a key section of the National Institutes of Health's guidelines covering recombinant DNA research, claiming that compliance under present procedures could lead to the disclosure of proprietary information to potential competitors.

The section in question is that which specifies the conditions under which certain experiments can be exempted from a prohibition on research involving more than 10 litres of culture—a situation which many companies are rapidly approaching as recombinant DNA technology moves from the research to the production stage.

Under an interim procedure agreed by the NIH's Recombinant DNA Advisory Committee (RAC) last month, applications for such exemptions have to be circulated to all members of the committee, and approved by a three-person subcommittee, before they can be granted by the director of NIH, Dr Donald Fredrickson.

Although legal provisions exist covering what may or may not be divulged by RAC members as "special government employees", Dr Fredrickson has said that he cannot guarantee the complete confidentiality of information submitted to the RAC as part of this approval procedure.

On the other hand, regulations covering the use of recombinant DNA techniques in private industry which are currently being drawn up by the Food and Drug Administration—and which will provide statutory protection for proprietary information—are not at present scheduled to be on the statute book before February 1980.

In the interim, unless an alternative way is found of protecting such information, pharmaceutical companies and other groups pursuing recombinant DNA research who need to go beyond the 10-litre limit as a step towards full-scale production, but do not want to divulge too much information to a potential competitor, have been faced with some hard choices.

Some, such as the Berkeley-based Cetus Corporation which is required by local laws to observe the NIH guidelines, plan to hold back on any experiments for which providing full details to the NIH would, they feel, prejudice their proprietary rights.

In contrast, the San Francisco firm Genentech is planning to take what one pharmaceutical industry official calls a "calculated risk" in conducting scaled up experiments according to the technical criteria that have been laid down by NIH, but without seeking the NIH's formal approval (although NIH will be informed when such experiments take place).

"To get the full benefits of this technology out, it is important to go rapidly to volumes greater than 10 litres", Mr Robert A. Swanson, president of Genentech, told *Nature* last week. He added, however, that there was no mechanism for doing this through NIH at present "that would take into account the necessity to protect proprietary information".

(Eli Lilley, the pharmaceutical company which has entered an agreement with Genentech to develop techniques for producing insulin, has suggested to the FDA that its proposed regulations—which are likely to require companies to show that a product has been developed under the NIH guidelines—"should include provisions for exempting studies commenced prior to the effective date of the guidelines or regulations".)

Issues related to the confidentiality of information, although they have been heatedly debated over the past two years in Britain's Genetic Manipulation Advisory Group, have tended to remain on the sidelines within the US debate, where greater attention has been given to public concern about the potential health hazards of the research.

As the production of substances such as insulin, growth hormone and interferon using recombinant DNA techniques comes closer to reality, however, companies which have agreed to comply voluntarily with the NIH guidelines are becoming increasingly concerned about being required to divulge commercially valuable information.

"There are not many companies wanting to do so at the present time, but within the next year or two you will have a lot of companies wanting to scale-up their research. And how you deal with proprietary information in such circumstances is causing some concern", says Dr Herbert Weissbach of the Roche Institute of Molecular Biology.



Dr Donald Fredrickson, Director of the NIH: under pressure on confidentiality

These sentiments are echoed by Dr John Adams, vice-president of the Pharmaceutical Manufacturers Association in Washington. "We do not feel that the NIH guidelines in their current form adequately address the problems that we have with regard to protecting the confidentiality of information. The problem is that there is nothing in the statutes of NIH that covers trade secrets."

At the same time, however, trade union and public interest groups are arguing that the more recombinant DNA techniques become part of standard production techniques, the greater the potential for serious health effects—and hence the greater the need for information about the processes involved.

Under the terms of the guidelines published by NIH last December, experiments requiring more than 10 litres of culture are prohibited unless the recombinant DNA's are rigorously characterised and "the absence of harmful sequences established". In such cases, the guidelines state that the experiments can be authorised by the director of NIH "under procedures specified by the RAC".

Exactly what these procedures should be is now under debate. At a meeting of the advisory committee held in Bethesda last month, Dr William Gartland, head of the NIH's Office of Recombinant DNA Activities (ORDA) suggested that as an interim procedure, applications for scaled-up experiments should be reviewed by the chairperson and two other members of the RAC.

This group, he suggested, would recommend whether the request should be approved by ORDA or whether it required review by the full committee, in which case a subcommittee of the RAC would review the application prior to it being passed to the full committee.

Various members of the committee objected to this procedure, claiming that all members of the RAC should

receive details of all applications for scale up. "Otherwise the sub-group could theoretically be given a blank cheque to pass experiments without reference to the full committee" says committee member Dr Karim Ahmed.

After a certain amount of debate, it was agreed that until the next meeting of the advisory committee in May, any application for experiments over 10 litres would be examined by a three-person subgroup, but would also be circulated to all members of the committee one week prior to the subgroup's meeting (so far no such application has been received by NIH).

The next RAC meeting will also consider the report of a subcommittee set up by chairperson Dr Jane Setlow on how the phrase "the absence of harmful sequences" should be interpreted. Some have argued that even an insulin producing gene might be described as potentially harmful, depending on where it was functioning; and Dr Setlow is keen to ensure a reasonable uniformity of interpretation between different institutional biohazard committees (IBCs), which are already required to make such judgements in certain circumstances.

The major question regarding procedure, however, is whether the pharmaceutical companies, if they are required by the FDA to comply with the NIH guidelines, are prepared to accept the proposed arrangements as adequate to protect proprietary information; and conversely whether an alternative arrangement more to their liking will be acceptable to all members—and in particular the "public interest" representatives—on the advisory committee.

Already at least one company has approached Dr Fredrickson to see if he could give his approval to certain scaled-up experiments without consulting the RAC. But Dr Fredrickson has informed them that, under his interpretation of the current guidelines, there is no way this can be done.

The pharmaceutical companies are now focusing attention on the form of regulations which the FDA is likely to bring in, following its announcement last December that it was proposing to require that any firm seeking approval of a product "requiring the use of recombinant DNA methods in its development or manufacture" demonstrate its compliance with the requirements of the NIH guidelines.

According to FDA Commissioner Dr Donald Kennedy, the agency is confident that it can find some way to protect proprietary information as it does now with other commercial products, even if this information has to be submitted to NIH for approval. But the drug companies are not so confident, and indeed are challenging

whether the FDA has the right to regulate the process, in addition to the product, of research.

"It may be difficult to show that any particular research protocol involves a specific trade secret", says Dr C. W. Pettinga, executive vice president of Eli Lilly which has been saying for some time that the need to conduct experiments involving more than 10 litres of culture is imminent, and is keen to press ahead with its own insulin research. "Also it may be possible for a competitor to determine from a series of protocols the general thrust of research activity that in part or in whole might be valuable proprietary information."

In comments to the FDA which Dr Adams of the Pharmaceutical Manufacturer's Association says are broadly supported in the industry, Dr Pettinga warns that unless information can be submitted in confidence to the members of the RAC, there is the danger of the premature disclosure of a potentially patentable idea "which would almost certainly preclude patent protection in most foreign countries".

Scale-up a local problem

He suggests that authority to increase the 10-litre limit, in a manner consistent with appropriate containment standards, be given to local institutional biohazard committees (which would also be empowered to approve the utilisation of host-vector systems and research protocols for all except experiments requiring the highest level of containment).

In broader terms, Dr Pettinga, with the support of other members of the PMA, says that "the regulation of basic research by FDA is not desirable". And he suggests a mechanism should be developed for voluntary compliance with NIH guidelines, and that FDA should remain involved solely

with procedures regarding the safety and efficacy of drugs, and not the regulation of research.

How FDA and NIH respond to these recommendations remains to be seen. Already there are disagreements over what constitutes adequate protection of potentially valuable information. NIH, for example, has suggested that to defend proprietary rights, institutions should apply for a patent before submitting information to the RAC; the pharmaceutical companies have replied that in most cases this is unlikely to work, since a potentially patentable discovery will be based on work that requires registration and/or approval before it can be carried out.

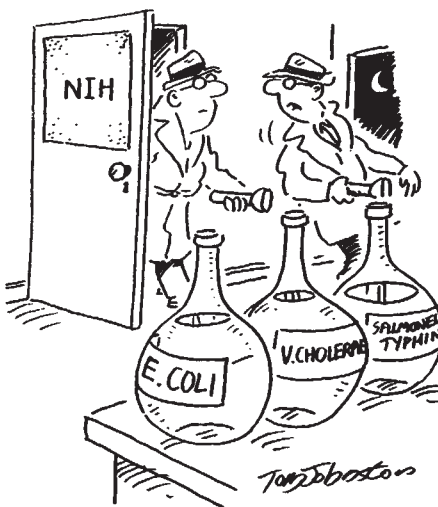
All sides are agreed, however, that the current situation is unclear. "We are hoping that somewhere down the road the rules of the game will be clarified and that reason will prevail. At present it is total confusion as far as we are concerned", Mr Peter Farley, vice-president of Cetus, said last week.

"Within the year we will be very concerned about the precise scale-up requirements. We will then have to pick and choose on a case by case basis whether to disclose full information of our plans to the NIH; if we were doing something very clever, the details of which we did not want others to know about, then for the time being we would do nothing."

One issue to be decided is how much information would need to be divulged to the NIH before a certain experiment would be approved as safe. "The characterisation of the gene presents no problem at all, since we would want to do this for our own safety. But describing specifically what one has done or providing the justification for going to larger volumes could involve sensitive information" says Dr Joseph Grady, of Upjohn Co.

A further matter of concern is that if private companies find they are unable to operate with the desired secrecy under NIH guidelines, then they may carry out their research in other countries with less stringent requirements. Already some US corporations with interests in recombinant DNA research are supporting the activities of foreign companies.

Any attempt to exploit loopholes in current legislation, however, is not likely to be looked upon kindly by Congress, which last year held back from legislating on recombinant DNA research in the private sector largely on the basis of companies complying voluntarily with the NIH guidelines. No company has refused to comply with the technical aspects, covering for example biological and physical containment levels; but neither has any said that they can work with the complete guidelines as they now stand. □



"Be careful! This place may be bugged!"

Britain's shadow science minister believes in experts

With the present government in Britain under extreme pressure to call an election, **Robin McKie** interviews the Conservative spokesman on science and education, who feels that the major research companies should be more open about the research they undertake. But expert committees should be left to experts

ASK a group of working scientists to react to the name of Mark Carlisle and it is likely that their responses will include a fair number of vague and inaccurate replies. This may not be particularly surprising but one cannot avoid feeling there should be a little more interest and reaction to a man who could shortly become overall leader of Britain's science effort.

In a surprise Shadow Cabinet reshuffle late last year, UK Opposition leader Mrs Margaret Thatcher appointed Mr Carlisle Shadow Secretary of State for Education and Science—in place of the previous, ebullient incumbent, Norman St John Stevas. Of course, the move does not automatically guarantee him the post of Education and Science Secretary in the increasingly likely event of the Conservatives bringing down the present Labour Government—but one only has to look to the example of Mrs Thatcher who held the same Shadow Cabinet post in 1970 and was appointed Secretary after Ted Heath won the general election that year. And it would also be strange to appoint another person just as Mr Carlisle had achieved some understanding of the complexities of the job.

So it seems timely to focus attention on the views of this 50-year-old barrister who may shortly influence so much of Britain's science policy. However, perhaps not surprisingly, Mr Carlisle is still reluctant to commit himself to particular doctrines or attitudes, stressing frequently that he will be guided throughout by the professional scientists and the various research advisory bodies. It is a natural reaction of a man new to the science arena, although one can already discern a less piercing or forceful interest in particular scientific topics than that shown by the present Secretary Mrs Shirley Williams, who has occasionally revealed great interest in subjects of special public concern such as genetic engineering.

However, some attitudes do become clear. In particular, Mr Carlisle shows a keen concern that science must be more closely tailored to suit the needs of British industry. In educational terms, this is reflected in a desire to increase emphasis on sandwich courses,

part-time education and polytechnic training.

And he particularly believes that greater encouragement must be given to further education at non-advanced, technician levels to provide necessary skilled manpower. "I think that one can foresee for the universities a period of consolidation, rather than expansion, and that any shift within the whole of education must go to scientific education and education in practical skills beyond the age of 16".

"The polytechnics have tended to move away from their original design and become less areas of technical education and more areas of general education. I think we have got to put the emphasis back on training the engineers and scientists that industry needs", he stated.

Mr Carlisle also stresses that more encouragement must be given to industry to carry out its own research rather than rely on strong Government backing all the time. "The Conservative philosophy is that Government should get off industry's back and leave it enough money for expansion which will allow more research. This will come from improving the tax structure and economic atmosphere of the country".

But if he is keen to tie science more closely to industry, then Mr Carlisle is less willing to have interference in the actual work of scientists. For instance he is quite adamant in his opposition to lay or union representation in bodies such as the Dangerous Pathogens Advisory Group or even the Genetic Manipulation Advisory Group.

"These are advisory boards on highly technical subjects and have to advise Government. If you are deciding on the appropriate degree of safety for testing then it seems to me that this is an area only in which the qualified are able to speak with authority", he added.

Instead Mr Carlisle believes, rather naively it may seem, that public opinion—"the most vociferous lobby in the country"—provides the best form of surveillance of the scientists' activities. However, on the subject of the Press, the major shapers of public opinion, he is less enthusiastic. "The newspaper reporting of science is probably no worse than the newspaper reporting of



Mark Carlisle: opposes union representation on GMAG and DPAG

anything else. It is only the bad news that makes the headlines".

But he does maintain that there should be greater openness in allowing more reporting of scientific matters. "I am quite sure that the major research companies have nothing to be ashamed of and should always be willing to speak to people and make it clear that they have nothing to hide."

On the more vexed problem of staff stagnation and ageing populations at universities, Mr Carlisle—in keeping with most other public figures—offers little in the way of comfort or solutions for the future.

"I am conscious that there is a feeling among younger members of university departments that today an awful lot of them are waiting to fill dead men's shoes. That is the root cause of the feeling of frustration among younger university lecturers.

"Sadly I don't think there is any simple solution. However, I think we have to invite the University Grants Committee completely to review the whole situation, particularly the question of levels of senior staff, and also encourage the setting up of fellowships, like the present SRC initiatives, to take up some of the strain".

And it is to be hoped that these measures, and the others outlined by Mr Carlisle, will prove in some way to be effective—for in a very short period they could be put into rapid practice. When, and if, that happens, we can then perhaps expect a little more reaction to the name of Mark Carlisle.

□

US urged to centralise scientific assessment of cancer risks

A SINGLE scientific organisation responsible for identifying and characterising potential carcinogens across a range of government agencies has been recommended in a report prepared by the staff of the US Office of Science and Technology Policy.

Such an organisation, which it is suggested should be based on an expansion of the national toxicology programme established last year by the Department of Health, Education and Welfare, would have two main responsibilities: to identify those substances which pose a potential carcinogenic risk to humans, and to characterise in quantitative terms the nature of the risk and the degree of certainty of current estimates.

The report is part of an attempt by the administration to improve the scientific base of the regulatory process, a need which President Carter was expected to refer to briefly in a speech on regulatory principles which he was due to give this week.

Addressing a senate subcommittee last week, Dr Frank Press, the President's science adviser, said that one of the current needs in science policy was to carry out an analysis of the research base of the regulatory agencies to see if it was adequate. "If more resources are needed, I cannot think of a higher

priority area than this one," he said.

Responding to a number of recent issues, such as heated debates over occupational exposure levels for lead and air pollution standards, the OSTP report says that in order to help develop a uniform decision-making framework for controlling potential chemical carcinogens, it is necessary to start by distinguishing scientific from regulatory decisions.

The first stage would involve scientific data collection and analysis, with risks expressed in comparative and quantitative terms, while the second would reflect a complete cost benefit analysis, taking into consideration human, economic and social factors—including the societal distribution on benefits and costs.

Priority for scientific analysis should be given to chemicals for which cost-benefit analysis performed with existing data suggests that regulation be indicated, but leaves too much uncertainty to justify immediate action.

"Priority setting and coordination of testing ideally should be done by a single group with input from all relevant agencies," says the report, recommending this approach in contrast to one in which assessment would be assigned to various scientific organisations on an agency-by-agency basis. □

UK ministry gives green light to 2,4,5-T

A REVIEW of the herbicide 2,4,5-T was published by the Ministry of Agriculture, Fisheries and Food (MAFF) last week. The document compiled by the Ministry's advisory committee on pesticides outlined the reasons for the MAFF announcement in July 1978 that herbicides containing 2,4,5-T were safe "if used in the recommended way for the recommended purposes" and that approval had been given for their continued use in the UK.

This is the advisory committee's 8th review of 2,4,5-T since 1967 and it points out that the decision it reached was based on certain premises. These are that "in general the dose makes the poison", that there can never be "total proof of safety", that where reasonable doubt about the risk of a product exists the advisory committee's policy is to "err on the side of safety".

The review points out that it is the presence of the toxic contaminant 2,3,7,8-tetrachlorodibenzo-p-dioxin (dioxin)—a known teratogen and carcinogen—which is central to the concern over the safety of 2,4,5-T, some 3 tons of which are used annually in the UK—1/15 of the 1970 figure. Pure 2,4,5-T, the advisory committee

concludes, offers no hazard to users. And it adds that the dioxin itself would "ensure no hazard" if it was present in the trichlorophenol used to manufacture 2,4,5-T in quantities less than 0.1 ppm.

In arriving at its conclusions, the committee reviewed several major studies on the herbicide, including a New Zealand Department of Health Safety Report which could find no link between human birth defects and 2,4,5-T spraying; an Australian National Health and Medical Research Council report refuting that birth defects in the state of Queensland were associated with the presence of dioxin caused by burning sugar cane stubble sprayed with 2,4,5-T; a West German Federal Health Office statement and Review each approving the use of the herbicide in the Federal Republic; and reports from Vietnam that 2,4,5-T spraying caused an increase in the incidence of malformation in children, but which were inconclusive owing to the inevitable inadequacy of the data from a war zone.

The advisory committee points out, however, that decisions such as this one on 2,4,5-T are never final. □

MRC unit finds UK suicide rate falls

THE MRC Clinical Psychiatry Unit in Chichester has released the results of a major study showing that from 1962-1974 suicide rates in the UK have declined in all age groups except young women of age 15-24. Cautioning against simple interpretations, Jim Jenkins, co-author of the report said that an "entire constellation" of factors must be taken into account in the case of the female figures including family size, number of children and patterns of female employment.

The overall decline in suicide rate could be attributed to the increasing social cohesion in Great Britain in the face of a declining standard of living and increasing unemployment. Ironically, the MRC unit itself is slated for unemployment. Following the Medical Research Council's usual policy of closing units when their directors retire the Chichester Unit will be closed in 1982.

An MRC site visit three years ago found that "suicide research had no future" and a petition to the MRC with the support of the MRC's National Staff Side Committee failed to re-open the case. "We tried to get the MRC to look at their decision again," says Jenkins, "but we were given no scientific explanation why the unit should be closed." □

Misha Voikhanskii finally permitted to leave Leningrad

DR MARINA VOIKHANSKAYA, the former Leningrad psychiatrist, was forced to emigrate from the Soviet Union in 1975 after she had repeatedly refused to administer neuroleptic drugs to political "patients". Her nine-year-old son, Misha was to have followed her, but his promised emigration visa was withheld until a few days ago.

Since coming to the UK in 1975, Dr Voikhanskaya has been an active campaigner against political misuse of psychiatry in the USSR. In 1977 she addressed the Honolulu meeting of the World Psychiatric Association on this subject. Meanwhile as Misha grew up he too became interested in psychiatric abuse. In December 1976, it was he who notified campaigners abroad that activist Vladimir Borisov had been taken to a psychiatric hospital.

Dr Voikhanskaya told *Nature* this week that she felt sure that the long delay in Misha's visa was an act of reprisal against her for having spoken out against Soviet abuses of psychiatry. "It is only due to constant pressure from the scientific community and the public at large that the Soviet authorities have finally given way." □

AGE SPECIFIC DEATH RATES FROM DIARRHOEA

Place & year	Age specific death rates per 100,000	
	0-11 months	1-4 years
New York, 1900	5603	398.7
New York, 1961	45	2.4
Punjab, India 1959	3446	312

(From Gordon et al., (1963),
Amer.J.Sci. 245 : 345)



A cure for a killer—but how to deliver it?

Millions of Third World children die of diarrhoea each year, although there is a simple cure available: oral rehydration. Discovered by scientists a decade ago, it has still not reached the people most in need. Anil Agarwal reports

THE possibility of oral rehydration has provided Third World health authorities with a very valuable breakthrough in the treatment of diarrhoeal diseases. Loss of fluids and electrolytic salts is the major complication in acute diarrhoeas, and if uncorrected, the continuing loss results in shock and eventually death. Until now rehydration has been possible only by administering fluids intravenously, as the small intestine loses its ability to absorb sodium during an attack of acute diarrhoea. Oral rehydration has now become feasible, however, with the observation that glucose absorption remains largely intact, and that sodium is carried along with it.

The application of this discovery—largely the result of work carried out at the Infectious Disease Hospital, Calcutta, the Cholera Research Laboratories, Dacca, Johns Hopkins University and the US Centre for Disease Control, Atlanta—now gives mothers the opportunity to treat their children suffering from diarrhoea themselves, by making a simple solution of glucose and electrolytic salts and feeding it to them.

Millions of deaths

Mothers no longer have to take their suffering children to health centres and hospitals tens of kilometres away, merely to seek the qualified nursing abilities required for intravenous treatment. For the majority of Third World mothers, who still do not have any reasonable access to health centres and hospitals, the necessity for intravenous treatment has really meant no treatment at all, and hence death for their children—a report from a research project situated in the relatively well-served region of Punjab, India, recently pointed out that though medical orders to village health workers called for children with clinical signs of dehydration to be immediately referred to the physician at the health centre, many children still died at

home of dehydration after they had been seen by the health worker. Many other children died without being seen at all. In this region, diarrhoeas were found to be responsible for 44% of all deaths amongst children less than three years of age. Considering that in many parts of the Third World, deaths amongst children constitute nearly half of all deaths in the population, the control of diarrhoeal diseases is of vital importance for the improvement of Third World health. It is estimated that there are some 500 million episodes of diarrhoea every year amongst children below the age of five. Experts differ, but it is widely accepted that these attacks result in the death of some 5-20 million children every year.

It is ironic that though diarrhoeal diseases have been reduced to a mere inconvenience in the developed countries, they still constitute the biggest cause of death and ill-health in the Third World. The primary cause of diarrhoeas is, of course, lack of clean water and generally insanitary conditions. But unfortunately these conditions are quite unlikely to change for at least another decade or two. WHO surveys show that as much as 78% of the Third World's rural population is still without clean community water supplies and about 85% is without adequate excreta disposal facilities.

Given these dismal conditions, oral rehydration has certainly opened up a glorious opportunity to take treatment, if not prevention, of diarrhoeal diseases to every doorstep in the Third World, however remote it might be, and control their worst consequences, dehydration and shock. In fact, oral rehydration can render an even more valuable service by helping to overcome the debilitation caused by the overwhelming majority of diarrhoeal attacks, which are mild and moderate, and do not lead to acute complications like dehydration. Together with mal-

nutrition, these diarrhoeas combine to form a vicious, mutually reinforcing complex of disease and ill-health. Malnutrition increases susceptibility to diarrhoeal attacks and persistent diarrhoeas increase malnutrition. If a child somehow survives the combined influence of these two, then a minor disease like measles often proves sufficient to kill him. However, studies now show that a child regularly treated with oral fluids should be able to maintain relatively good health. Trials in the Philippines for seven months, and in Turkey for 16, revealed that children who were given oral fluids had much higher monthly weight gains than those children who were left untreated: the mean weight gain of the non-treated children was significantly below that of the average growth curve.

Tests

The first serious test of oral rehydration took place in 1971 when cholera epidemics were taking a heavy toll of millions of Bangladeshi refugees who had poured into India. Two or three physicians, assisted mainly by the illiterate family members of the patients, were able to treat with oral fluids 3,700 severely ill refugees in less than two months. It was a state of total confusion, with up to 200 new patients being admitted daily; even space on the mud floor was full of purging patients. But only 125 patients (3.6%) died—and half of these before they could be given any fluids. By contrast, 9% of cholera cases in Italian hospitals died in 1973.

WHO and UNICEF are now spear-

Above: left, the death rate tells its own story. Right, the malnourishment of this child obscures diagnosis. The combination of symptoms is not rare in cases of diarrhoea, where repeated attacks worsen the condition. The majority of cases occur within the age group 6 to 18 months.



Measuring bottles and clean glassware are available in hospitals, as here in Papua New Guinea, but not in villages.

heading an international campaign to persuade Third World governments to introduce oral rehydration work in their primary health care activities. Numerous WHO-sponsored field studies are in progress, or have been completed in Egypt, El Salvador, Guatemala, India, Iran, the Lao People's Democratic Republic, Liberia, Nigeria, Turkey and the Philippines. Through these studies, WHO hopes it will be possible to convince local health administrators of the practicability and benefits of the new method. Many Third World hospitals have already adopted oral rehydration. The great majority of cholera patients at the Infectious Diseases Hospital in Calcutta, for instance, are now being treated in this way. The switchover from intravenous to oral fluids has meant a saving of about 50,000 dollars every year for the hospital in the cost of the fluids alone. More than a third of the beds in children's hospitals and wards in developing countries are usually occupied by diarrhoea cases receiving expensive antibiotics and intravenous fluids, thus putting a heavy load on their limited budgets. These units will greatly benefit from oral rehydration.

Delivery

Exactly how this new revolutionary therapy should be delivered to the Third World's million villages is, however, a matter of controversy amongst child health experts. WHO believes that the ideal course of action, in the interest of standardisation and quality control, is to prepare packets of oral rehydration salts in a standard mixture (3.5 g sodium chloride, 2.5 g sodium bicarbonate, 1.5 g potassium chloride and 20 g glucose) that would make a litre solution, and then sell these packets widely through commercial channels or distribute them through rural health centres. UNICEF is helping developing countries not only to distribute these packets but also to set up units to produce them. The packet approach is gaining acceptance. UNICEF has already distributed more than a million packets in developing countries, while Royal Drugs Ltd in Nepal has marketed over a quarter of

a million packets through commercial channels in the Kathmandu Valley. Since 1974, Indonesia has been running a national rehydration campaign with provision of locally packaged glucose-electrolyte mixture to its more than 2,000 rural health centres, and large numbers of commercial firms also retail the packets in the open market place. Costa Rica has instituted a national programme using packaged solutions, with the help of the Pan-American Health Organisation. The Cholera Research Centre in India has launched a drive to popularise oral fluid therapy among doctors and the lay public. Under contract from the Indian Council of Medical Research, a reputable drug firm has been entrusted with the large-scale production of oral rehydration packets.

WHO's "appropriate technology for health" programme is simultaneously trying to find cheap and simple packaging methods, in the interest of the poorer consumers: packaging constitutes as much as 50-75% of the price of producing a pre-packaged mixture. Machine-packaging is one possibility, using a vacuum sealed aluminium packet, coated with a plastic membrane inside. This type of packaging would minimise the moisture content, thus increasing the shelf-life of the powder. The price of a 1-litre machine-packaged UNICEF packet is currently estimated at 6.5 US cents. Hand-packaging would be cheaper and would create more employment, though the shelf-life of the powder would be less. In many rural areas of the Third World there already exist small packaging workshops where household articles and some drugs are packaged into polythene bags, and such workshops would be ideal for the hand-packaging of glucose-electrolyte mixtures. The cost of such mixtures is estimated to be about 5 US cents. All the ingredients could be put together in one polythene packet, or, because hand-packaging retains some moisture, the salts could be packaged separately and then added to a packet containing glucose. The cost of the latter technique would be higher, but the shelf-life would be increased.

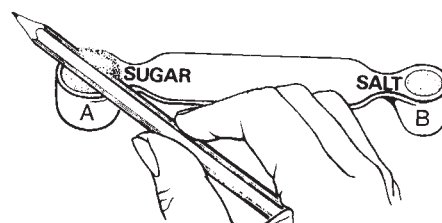
The major advantage of pre-packaged solutions is that mothers would get the quantity of each ingredient correctly measured. An excess of potassium salts can be toxic. Large excess quantities of sodium can cause hypernatraemia, a condition that affects the heart.

Critics

There are, however, several critics of the WHO-UNICEF approach. Their arguments are largely based on the grounds that this approach has not adequately taken into account the extent of poverty of a large percentage

of the rural population and on the logistic difficulties of distributing centrally-produced packets to every village in the Third World. Packaging, which only adds to costs, can make even this simple and cheap oral rehydration therapy virtually prohibitive for millions of mothers. Distribution through commercial channels increases the price further by adding profits for manufacturers, distributors and retailers. Although these packets will eventually be required in hundreds of millions, manufacturers have not leaped into production, because, it seems, of the low profits at which they would have to sell them to reach the mass of the consumers. Distribution through health centres would also not reach the majority of the Third World rural population. In Indonesia, for instance, it has been estimated that distribution of packets through health centres has reduced mortality to less than 5% in dehydrated children, but only 10% of the total cases actually use health centres.

Another problem with the WHO standard 1-litre solution is that in most villages of the Third World mothers will not find it easy to measure a litre. Instead, every culture has some standard measure, based on a readily available container: in India, for instance, a 300 ml beer bottle can be found almost everywhere. But packaging in smaller quantities will only increase the cost of packaging, and hence the total cost of treatment. Moreover, keeping a litre of aqueous solution



This simple measuring spoon was developed for home use in Indonesia.

containing glucose for more than a few hours is not advisable in unhygienic rural conditions, as it would provide an excellent culture for several pathogenic organisms. Instead, it would be better to make the solution every time it is needed.

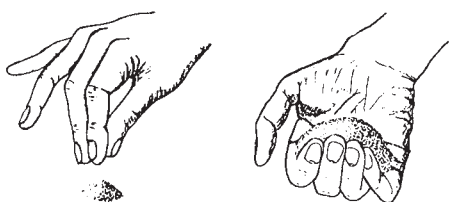
Mother the focus

The alternative approach being suggested is to make the mother the focus of oral rehydration treatment—to enable her to make and administer the solutions herself. Jon Rohde, who is working with a Rockefeller Foundation project on oral rehydration in Indonesia, argues that dehydration is just a form of malnutrition: fluid-electrolyte malnutrition. The key role of the mother in the treatment of

protein-calorie malnutrition is widely accepted. "Her role in replacing fluid and electrolytes is just as crucial," says Rohde, "perhaps more so considering the acute course of fluid-electrolyte malnutrition."

To enable the mother to treat her child herself, the WHO standard mixture can be considerably simplified. One problem is the availability of glucose, which has to be imported by most developing countries. Rural mothers will find it very difficult to obtain it. Glucose could therefore be replaced by the easily available white sugar (sucrose) which breaks down to glucose in the intestine. Sugar-salt solutions have indeed been tested by several researchers and have been found to be almost as effective as the WHO standard mixture in helping acute diarrhoea patients to recover. The simplicity of the sugar-salt solutions makes it possible for virtually every mother to become self-reliant. If nothing else is available, Dr William Cuttings of the London School of Hygiene and Tropical Medicine even recommends that mothers would do very well to use brown sugar or honey, which is available in many rural areas where beekeeping is a traditional practice. For potassium a mother could possibly squeeze in some orange or any other citrus fruit.

Researchers at the Institute of Child Health, Lagos, have found that poor mothers adopt the sugar-salt solution with great alacrity. All the



Fingers alone can measure quantities roughly. Left, 1.5 g, right, 30 g.

women attending the Institute's clinic are being taught oral rehydration therapy. In a recent survey, even women expecting their first child and others who had never used the service were able to describe the treatment. The survey however revealed that nearly half of the 217 women interviewed were planning to use more than four times the quantity of salt suggested to them (one-quarter teaspoon in a standard local beer bottle filled with water) and three cases were going to use sixteen times as much. Even very educated Western mothers have the tendency to believe that if something is good, more of it is even better.

To solve the problem of accurate measurement, a special plastic spoon has been developed in Indonesia which

has two separate indentations for measuring salt and sugar. This plastic spoon costs just 0.5 US cents. Such spoons are not only cheap, they could easily be made to suit local measures. Jon Rohde is using these spoons to teach children in primary schools and groups of mothers to make sugar-salt solutions. Dr Zafarullah Choudhury, a leading primary health care innovator from Bangladesh, is also teaching schoolchildren to make their own solutions. Because of its simplicity, Rohde strongly argues oral rehydration should be made everybody's business: "Teachers, agriculture extension workers, government employees, boy scouts, women's groups and even schoolchildren should be taught to mix rehydration fluids and use them."

No global method

Dr Christopher Lomax of the London-based Appropriate Health Resources and Technologies Action Group argues that "as in most 'appropriate technologies' it is difficult to recommend a single method to be used on a global scale, for each method will be viewed from a different perspective in different parts of the world".

Dr David Morley of the Institute of Child Health, London, who also strongly supports the use of spoons for measuring, believes that it would be best if national oral rehydration programmes were designed to operate at different levels of sophistication. At the level of the health centre, where even seriously dehydrated cases can be expected to turn up, both oral and intravenous therapy should be provided using the WHO standard mixture. But at the level of the individual mother, who has to treat most of the time mild and moderate diarrhoea cases, health authorities should promote the use of simple sugar-salt solutions made with the help of spoons.

WHO's Director-General, Halfdan Mahler, has in his speeches favoured the mother-based approach as best both in the long and short term. For WHO's new community-based approach to primary health care to succeed, Mahler has argued that a demystification of medical technology is needed. Oral rehydration, he believes, is an extremely good example where the responsibility of the community's health can be put into the hands of the community itself.

Maintaining standards

WHO's officials, however, who are very conscious of the organisation's reputation for medical standards, find it necessary to be cautious. WHO does not oppose any studies to test the efficacy of home-made sugar and salt solutions. But as all the major studies that have been published to date on the



The trend to bottle-feeding among the urban poor increases risk. Poverty means the mixtures are over-dilute, leading to malnourishment, and sterilisation of the bottles is impossible.

efficacy of oral rehydration have used the complete formula, WHO is making all attempts to promote widespread distribution of packets containing the standard mixture. It is felt that with sufficient education by health auxiliaries the complete formula can be mixed by mothers at home. With recurrent use of this solution, less education would be needed, and therefore hopefully there will soon be a minimum need for home-made sugar-salt solutions.

Dr Richard Cash of Harvard University also opposes attempts to simplify the oral rehydration therapy too fast. He believes that the medical community should not be bypassed. It is possible to go to the people directly and teach them self-help, but local doctors too must be adequately convinced of the effectiveness of a therapy before it is widely promoted. Otherwise, they will only discourage people from using them.

Furthermore, Dr Cash argues, the real problems in reaching the people is not scientific—whether one solution should be used or another—but lack of political will. It has taken ten years for governments to wake up to the possibility of oral rehydration since it was first indicated by scientific research. Producing packets and selling them is a far simpler job than undertaking a massive programme to educate every mother about oral rehydration. Governments, which still have to pick up the courage to provide more than half their population with adequate health services, are more likely to adopt the easier alternative.

The debate about oral rehydration does show that it is often easier to generate new medical knowledge that meets the needs of the neglected, underserved poor of the world, than it is to find ways to enable them to benefit from that knowledge. The debate about the delivery of oral rehydration parallels virtually every issue that has been raised in the more general primary health care debate about delivering health services to the rural Third World. □

news in brief

France and Germany launch millimetre radio telescope project:

The final go-ahead of the joint Franco-German millimetre radio telescope project was announced by Dr Kjell Johnson at the Rome meeting of the European Physical Society on 26 March. Conceived in 1973 the project was approved by the Centre National de la Recherche Scientifique (CNRS) and the Max-Planck-Gesellschaft zur Förderung der Wissenschaften (MPG) at the end of last year. CNRS and MPG will share the initial construction expenses of 160mFF equally with much of the MPG contribution coming from the Volkswagen Foundation. The facility will consist of a single fully steerable 30 m dish located at an altitude of 3,000 m in the Sierra Nevada in the south of Spain near Granada and an interferometer of three 12–15 m fully steerable and moveable antennae with a baseline of 1.5 km x 0.8 km located at 2,500 m in the Plateau de Bure near Grenoble. In exchange for offering a site and a local headquarters in Granada, Spanish astronomers will get about 10% observation time at both observatories. The main office of the Institut de Radio-astronomie Millimetrique (IRAM) in Grenoble will house 20–25 people by the end of 1979. The construction order for the large dish is ready to be placed and design of the interferometer is in the initial stage with the dish expected to be operational in 1982 and the interferometer in 1986. Millimetre range astronomy covers cold thermal sources (a 10° blackbody peaks at 1 mm wavelength) including planets, asteroids, and dense interstellar molecular dust clouds.

Costs may boost UK energy conservation: An inter-Departmental report by officials under the chairmanship of the Department of Energy declares that higher prices will “provide scope for considerable further savings” as the price of oil in particular is expected to double by the end of the century. Higher prices form a principal part of the government’s recommended strategy and it stresses that economic energy pricings should “reinforce conservation measures and not work against them.” This is believed to be a reference to the relatively low price of petrol in the UK which many politicians would like to see raised. A Department of Energy spokesperson said that “the Government should not take measures to cut fuel prices.” Other measures in the government’s strategy include: information; “a good example from Government”; and specialised advice and training. Research and development, expected to rise to £30m per year, is believed to be adequately funded at present. There are to be no mandatory controls on industry, which consumes the largest share (39%) of the UK’s energy budget. The large energy users (50% of industrial energy is consumed by 14 firms and 70% by less than 200 firms) are believed to have an incentive to save energy voluntarily and the report argues that “mandatory measures would increase the burden on industry and would operate against the immediate need to increase industrial efficiency and competitiveness.” Energy Conservation: scope for new measures and long term strategy. Energy Paper Number 33. HMSO. £2.

Europe moves further towards independent space capability:

The remote sensing board of the European Space Agency (ESA) approved, on 15 March, the start of a remote sensing preparatory programme to develop the technological systems necessary to monitor both land and ocean surfaces. The preparatory programme will be for two years with the sizeable budget of nine million accounting units. (The 1979 ESA budget is 573 MAU with large individual programmes such as Exosat and the space telescope costing 25 MAU

and 15 MAU respectively.) Denmark, France, Italy, Netherlands, Sweden, and the UK have already subscribed and other member states are expected to join shortly. The programme will depend heavily on independent national development programmes as well, such as the multi-mission platform being developed in France and the synthetic aperture radar system being developed in Germany, both of which are being prepared for use in Spacelab flights in 1983.

Moscow Radio criticises US scientists:

US scientists participating in the growing “SOS” (Scientists for Orlov and Shcharanskii) campaign has been severely censured by Moscow Radio’s English-language service to North America. The 2,000 or so signatories of the SOS protest pledge themselves to refrain from taking part in certain specific forms of exchange programme with the Soviet Union until Orlov and Shcharanskii are released and cleared of the charges of anti-Soviet activity for which they were sentenced last year.

According to Moscow Radio, the SOS participants are misinformed and chauvinistic—the latter term since they are acting on the “assumption that only the Soviet Union will suffer” from such severance of ties. The commentator stated that, on the contrary, research from Tokamak and “many other achievements of Soviet science” are used in US laboratories, and that in any case, all blockades—political, economic and diplomatic—of the Soviet Union have failed in the past. This broadcast took place on the same day that a group in Britain announced the formation of a “Lerner Circle” to campaign for Aleksandr Lerner, the cyberneticist and a “refusnik” of eight-year’s standing to be granted an emigration visa. Among the founding members of the “circle” are Max Perutz, Nevill Mott and Rodney Porter.

US to increase scientific cooperation with Brazil:

The United States is to expand its scientific cooperation with Brazil as part of an effort to increase the relations between the two countries, Vice-President Walter Mondale said on a visit to the country’s capital, Brasilia, last week.

Mr Mondale said that the Brazilian president, General Joao Baptista Figueiredo, had accepted an invitation to send the president’s science advisor, Dr Frank Press and a team of specialists to consult with Brazilian scientists on the form that this cooperation might take.

Little was said during the Vice-President’s visit of previous US criticism of Brazil’s nuclear power programme, in particular its plan to import reprocessing facilities from West Germany as well as nuclear plants.

New Windscale leak discovered:

Borehole sampling in the vicinity of a temporary storage tank at British Nuclear Fuel Ltd’s reprocessing plant at Windscale, Cumbria has shown a marked increase in radioactivity. The temporary storage tank holds highly active liquid wastes dissolved in nitric acid for concentration by evaporation after which the waste is transferred to highly active waste storage silos. A BNFL spokesman said that the activity in the borehole was 1% of that in the temporary tank which in turn was 1% of that in the highly active tanks but said that the precise level of activity was “unavailable.” Borehole monitoring was initiated two years ago when 200 gallons per day of liquid waste was found seeping into the soil around one of the concrete storage silos. This leak has not been located but the area has been isolated and the radioactivity is believed to be retained in the soil, as tests of ground water have shown no increase in activity.

correspondence

Nuclear decisions

SIR,—In a recent article (22 February, page 594) Peter Taylor calls for changes in the way in which decisions are taken on nuclear energy matters. The article contains two straightforward issues and two more complex ones of interest to the Health and Safety Executive. Let me first dismiss the straightforward points.

The first is the reference to the alleged assumption that the issues are "primarily 'scientific' and 'technical', and thus... the exclusive field of experts". I doubt if anyone really makes this assumption these days. It is certainly not a view held in the Health and Safety Executive.

The second issue relates to the Hinkley Point incident, which Peter Taylor has seriously misunderstood. The ministerial statement, to which he refers, makes it quite clear that the coolant involved was not the core coolant, which is carbon dioxide, but the sea water coolant for auxiliary equipment. Cooling was maintained by a jury-rigged system involving a fire pump. The reactor coolant was unaffected.

The most important of Peter Taylor's points relates to risk assessment. He casts quite legitimate doubts on the accuracy with which quantitative probability estimates can be made for various scales of accidents and their consequences. He goes on, however, to say that "probabilistic presentation obfuscates rather than illustrates the potential effects". On this I think he is wrong. The study of the range of accidents and consequences, including very serious ones, is helpful and is standard practice. The search for a worst case is less useful because of the difficulty of defining "worst". The worst case owes more to the imagination and inventiveness of its postulator than to reality. More seriously, the discussion of a worst case diverts attention and effort away from the more likely accidents. Moreover, a study of very serious accidents without reference to their likelihood does not provide any basis for decisions. Thus, for example, the worst possible accidents in many forms of transport, chemical processing and gas distribution would be unacceptable if they occurred frequently; it is the very low probability of these serious accidents that makes these industries acceptable in a society which relies heavily on the benefits they bring. It would be wrong to ban large passenger aircraft just because one might, one day, crash into a crowded stadium. It would be wrong precisely because the probability is very low, even though the consequences would be very severe.

Of course the probability and scale of accidents do not alone provide a definitive basis for decisions on, in particular, the licensing of nuclear installations. They do, however, provide an essential input.

The final point of importance relates to the supply of information. Peter Taylor writes of "a retreat into secrecy" but the alternative, moving towards greater openness, is not without difficulties. Much of the information in the detailed safety

assessment of a plant is owned by the designer and is of real commercial value to him. So long as we expect to have a commercial structure, nationally or internationally, to our nuclear industry this point will limit disclosure. I believe, and have said so in public, that there is too much use of the claim for commercial value and that we ought to move towards a more open situation. But this requires additional resources, both in the industry and in the Executive, and my hand is not strengthened when published information is misquoted and misused. The "selective use of the results of scientific research" is not confined to any one group.

H. J. DUNSTER
Health and Safety Executive, London.

SIR,—Peter Taylor (22 February, page 594) refers to the safety target which I proposed in 1967. He is right in quoting my view that the setting of a target in no way gives assurance that the target is or will be achieved.

Both the Flowers Report and the recent report of the Lewis Commission (USA) strongly support the use of fault trees and event trees for licensing purposes and to direct the research programme. Lewis also supports the Rasmussen methodology. Use of these techniques necessarily requires an adequate data base.

The Safety and Reliability Directorate (SRD), originally under my direction, have worked extremely hard during the last 13 years to collect relevant data and develop the highly disciplined and critical assessment techniques. These are now used in the nuclear industry and are being more widely used in the chemical and allied industries in association with over 60 agencies and major companies in over 16 countries.

I know that numerical assessment is not perfect and is not enough in isolation to determine design and operation but, rather than obfuscate, the need to obtain data and analyse quantitatively wonderfully sharpens the mind and illuminates the problem, even if to show up its difficulties.

In my view the use of emotive words such as credible, incredible, maximum credible, did much to confuse safety issues in the first 10–15 years of reactor design.

Taylor asks that the worst condition should be stated. There is seldom a "worst". By assuming a combination of more and more unlikely events I am sure that I could develop a hypothetical situation to beat Mr Taylor, but of what value would this be?

When referring to the AGR and the NRPB/NII study Mr Taylor quotes 100,000 and 600,000 casualties. The reader might reasonably believe this to be deaths, but paragraph 266 of the Flowers Report clearly states that the major effect would be thyroid damage. The number of deaths were estimated to be around about 300/400 although this number could be an order of magnitude higher or lower.

As to the fast reactor, the Flower's Report (paragraph 304) states, "The consequences of such accidents would be so severe that the FBR could scarcely

be contemplated on an extensive commercial basis unless it is established with a very high degree of confidence that reactivity accidents can be controlled so as to prevent gross fuel vaporisation." It is still my view and that of my colleagues that gross fuel vaporisation is highly improbable, and many international research programmes still continue to provide further evidence on this point.

I support Mr Taylor's desire to ensure a highly critical approach on safety matters. We have done much to improve the standard of objective criticism in the UK, but I invite Mr Taylor to be equally concerned about the safety of man and the environment in all our industry.

F. R. FARMER
(Safety Adviser to the UKAEA)
*United Kingdom
Atomic Energy Authority.*

SIR,—Peter Taylor (22 February, page 595) states "... We were able to run NRPB calculations (which were for a notional site) for a specific locality (we chose Kalkar in West Germany). We discovered that NRPB had chosen not to present their own worst calculations—the results of one weather category which led to 600,000 casualties. Our site-specific study, having altered only the population data on the programme, produced over 900,000 casualties". Perhaps this concise account disguises the actual situation and I should like to reply in the absence abroad of Dr G. N. Kelly, the principal author.

When NRPB published its initial study of consequences of notional fast reactor accidents (Kelly, G. N., Jones, J. A. and Hunt, B. W., An estimate of the radiological consequences of notional accidental releases of radioactivity from a fast breeder reactor, NRPB-R53), it stated that "further analyses are being undertaken to assess the sensitivity of the results to variations of the more important parameters". At that stage no calculation of the type referred to by Mr Taylor had been done. In fact, the first sensitivity analysis has just been published (Kelly, G. N., Jones, J. A. and Simmonds, J. R., The influence of the physico-chemical form of the aerosol on the radiological consequences of notional accidental releases of radioactivity from a fast breeder reactor, NRPB-R73). Further studies are in hand which will include variation in meteorological conditions and population densities, from the likely parameters used in the initial study to the remote but nevertheless possible extremes. It is notable that the Political Ecology Research Group itself showed (RR-1, A study of consequences to the public of a severe accident at a commercial FBR located at Kalkar, West Germany) that the increased magnitude of the consequences in the worst conditions is broadly compensated for by the lower frequency of occurrence, giving comparable risks.

When NRPB has completed its investigations they will be published through HMSO.

M. J. GAINES
National Radiological Protection Board.

news and views

Opioid peptides and their relatives

from John Hughes

MORPHINE appears to produce its analgesic pharmacological effects by mimicking the action of natural endogenous opiate peptides. Recent research has illuminated various aspects of the origin and physiological relevance of these peptides.

The critical structure responsible for opiate peptide activity is Tyr-Gly-Gly-Phe(X) where X is a hydrophobic amino acid such as methionine or leucine in Met and Leu-enkephalin. Either N or C-terminal extension of these sequences preserves opioid activity to some degree although an N-terminal block, acylation, for example, abolishes activity. H. R. Morris originally noted the homology between the β -lipotropin (LPH) sequence 61–65 and Met-enkephalin, and the relationship between LPH, Met-enkephalin and β -endorphin (LPH 61–91) has since received considerable attention. All fragments of LPH commencing with residues 61–65 have opioid activity to some extent although peak activity is seen with Met-enkephalin and the C-terminal fragment of β -endorphin. This has posed problems in determining the possible biological significance of a given sequence, particularly since a number of active fragments of LPH have been isolated from pituitary extracts. However, it now seems likely that proteolysis during extraction accounts for the presence of most of the α and γ -endorphin (LPH 61–76 and 61–77) in pituitary extracts (Rossier *et al.* *Life Sci.* **21**, 847; 1977). LPH 61–77 (γ -endorphin) may in fact be the first metabolic product of β -endorphin allowing the final inactivation by amino peptidase which does not attack intact β -endorphin.

There is now overwhelming evidence that a family of related lipotropin/corticotropin intermediate lobe peptides are derived from a common glycoprotein precursor of molecular weight $\sim 30,000$ (Mains, Eipper & Ling *Proc. natn. Acad. Sci. U.S.A.* **74**, 3014; 1977; Roberts & Herbert *Proc. natn. Acad. Sci. U.S.A.* **74**, 4826; 1977). Bio-

synthesis of β -endorphin from β -LPH and its precursor (pro-opiocortin) has been demonstrated with radiolabelled pulse chase experiments in the pars intermedia (Crine *et al.* *Proc. natn. Acad. Sci. U.S.A.* **75**, 4719; 1978) and there is evidence that corticotropin and lipotropin/endorphin activity is present in the same secretory granules (Weber, Voigt & Martin *Brain Res.* **157**, 385; 1978).

The mRNA encoding for pro-opiocortin has now been purified from bovine pituitary intermediate lobe (Nakanishi *et al.* *Proc. natn. Acad. Sci. U.S.A.* **75**, 6021; 1978) and a further report in this issue of *Nature* (Nakanishi *et al.* page 423) now describes the full nucleotide sequence of this mRNA following cloning of the cDNA species in *Escherichia coli*. Inspection of the corresponding amino acid sequence reveals a number of interesting features. The molecule itself seems to consist of four repetitive units based on the MSH/ACTH core sequence, these units are separated by paired basic residues. ACTH and β -lipotropin exist as contiguous peptides at the C-terminal and are separated by the Lys-Arg sequence. Thus, in common with other protein precursors, the biologically active sequences are flanked by paired basic residues at which proteolytic processing takes place. An exciting and novel aspect of this study is the recognition of a new MSH sequence termed γ -MSH, this sequence is also flanked by paired basic residues and by implication could be of physiological significance. Nakanishi *et al.* postulate that the structural gene for pro-opiocortin may have evolved through a series of sequence duplications of certain common ancestral DNA segments, other modifications occurring through substitution, addition or deletion of some regions.

The existence of so many potentially active molecules in one precursor is a subject of much interest. The question of how pro-opiocortin cleavage is regulated is thus an intriguing and important one. Are all the possible

peptide sequences processed and released or do controlling and inactivating mechanisms exist? Relatively little is known of these mechanisms and the enzyme(s) responsible for sequence cleavage remain to be isolated. There is evidence that these control mechanisms may alter during development (Silman *et al.* *Nature* **276**, 526; 1978). Thus α -MSH, CLIP (ACTH 18–39), β -MSH and β -endorphin predominate in the fetal pituitary of the rhesus monkey whilst ACTH, γ -LPH and β -LPH predominate in the adult. Similar changes in these patterns of peptide production may occur in the adult in response to pathological states or changes in hormone balance. The identification of N-acetyl forms of β -endorphin (Smyth *et al.* *Nature* in the press) may reflect one controlling mechanism whereby acetylation inactivates the opioid peptide whilst leading to activation of α -MSH. The activity of the acetylating system could thus determine the balance of biological activities between these two peptides and could provide another point of disturbance leading to altered neuronal activity and mental states.

Pro-opiocortin is a glycosylated peptide and glycosylation may be important for protection of the prohormone from nonspecific proteolysis during packaging and could also direct cleavage of specific sequences through conformational effects. A recent paper by Loch and Gainer (*FEBS Lett.* **96**, 769; 1978) illustrates this view. Tunicamycin inhibits glycosylation and although it does not prevent synthesis of un-glycosylated pro-opiocortin there is a rapid degradation of this un-glycosylated species with the production of atypical peptides. Conceivably a disturbance of glycosylation in pathological states could lead to the production of 'abnormal' neuropeptides with undesirable side-effects on neuronal function.

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Despite the presence of the Met-enkephalin sequence in β -LPH there is no evidence that β -LPH acts as a precursor for methionine-enkephalin. Neurones immunoreactive to β -endorphin, β -LPH, ACTH and α -MSH have been found in the brain (Bloom *et al. Proc. natn. Acad. Sci. U.S.A.* **75**, 1591; 1978; Watson *et al. Science* **200**, 1180; 1978; Jacobowitz & O'Donohue *Proc. natn. Acad. Sci. U.S.A.* **75**, 6300; 1978). It is not clear whether this is one single neuronal system, although the localisation of cell bodies in the hypothalamus and their projections to other brain areas are very similar; however these neurones may be clearly distinguished from the more diffuse enkephalin immunoreactive neurones (Watson *et al. Nature* **275**, 226; 1978). As β -endorphin/LPH antibodies also recognise pro-opiocortin but do not react with enkephalin cell bodies or neurones, it seems unlikely that a common precursor is involved. Studies on the incorporation of ^3H -Tyr into the enkephalins have shown that these peptides are locally synthesised in gut and brain and that synthesis may be blocked by protein synthesis inhibitors (Sosa *et al. FEBS Lett.* **84**, 195; 1977; McKnight *et al. Proc. Roy. Soc. Ser. B* in the press). The nature of the enkephalin precursor(s) is therefore of considerable interest. Recent work has thrown

some light on this problem (Lewis *et al. Proc. natn. Acad. Sci. U.S.A.* **75**, 4021; 1978). Extracts of striatum contain proteins (molecular weight 40,000 and 100,000) that yield opioid peptides on tryptic digestion, these peptides can be distinguished from LPH fragments by high pressure liquid chromatography. These findings may explain the presence of unidentified opioid peptides in the brain and striatum (Hughes *et al. Br. J. Pharmac.* **61**, 639; 1977; Yang *et al. Neuropharmacology* **17**, 433; 1978) since they could be intermediate products resulting from the conversion of pro-enkephalin to enkephalin.

Kangawa *et al. (Biochem. biophys. Res. Commun.* **86**, 153; 1978) have identified a new peptide containing leucine-enkephalin in pig hypothalamus. This 15-residue peptide, rather unfortunately in my view termed α -neo-endorphin, contains the leucine-enkephalin sequence followed by the basic sequence -Arg-Lys-Arg-, an observation which supports the concept of a separate enkephalin precursor. Studies in our laboratory indicate the presence in brain of other opioid peptide sequences in much greater amounts than those found for α -neo-endorphin and the relationship between these peptides will be of considerable interest. Also, their existence sounds a cautionary note for those of us using immuno-

assay and immunocytochemical techniques in view of possible cross-reactivity and misinterpretation.

Enkephalin immunoreactivity has also been detected in cells of the anterior pituitary producing growth hormone (Weber *et al. Proc. natn. Acad. Sci. U.S.A.* **75**, 6134; 1978). Could it be that growth hormone and methionine or leucine-enkephalin share a common precursor? A common precursor for both enkephalins is also a possibility and this would explain the apparent coexistence of these peptides in the same neurones although this has yet to be demonstrated conclusively.

The application of recombinant DNA techniques is relatively new to this field but it is likely to prove a powerful and original approach. Nakanishi *et al.* suggest that the nucleotide coding regions for ACTH/LPH may have evolved by a series of genetic duplications since the cloned DNA insert for pro-opiocortin is characterised by unusually high C+G nucleotide base contents and also by numerous DNA sequence duplications. Other neuro-peptides also probably evolved in this fashion although nothing is known at present as to the origin of molecules such as substance P or neurotensin. The further unravelling of the various protein-peptide relationships is likely to prove both fruitful and exciting. $\square$

The representation of three-dimensional objects

from N. S. Sutherland

THE tasks carried out by the human brain are so complex that the only way in which to specify precise theories of how they are executed is to construct computational models. It is at present not possible to construct formal proofs to demonstrate what a complex but well specified series of information processing operations will accomplish. The number of operations needed to perform such tasks as the recognition of visual objects is so vast that we can only be sure that a given model actually accounts for successful performance if we translate it into a computer program and demonstrate that the program when run executes that task. Few, if any, such programs run successfully at the first attempt, but the discipline imposed by writing them and the study of the ways in which they fail generate insights into the structure of the task and the structure of the operations needed to carry it out.

A working computational model of task performance becomes a candidate for a theory of how the brain itself performs that task. Because of their complexity it is sometimes difficult to test such models experimentally, but in principle and with sufficient ingenuity they can be subjected to the same experimental tests and their plausibility evaluated in exactly the same way as the vague theories normally proposed by psychologists or the even vaguer ones sometimes advanced by physiologists.

In 1976, David Marr¹ proposed a computational model of how the brain recovers from the grey scale data present in the retinal image a two-dimensional description in which lines and edges are explicitly represented: he called this description 'the primal sketch'. The processes suggested by Marr are extremely complex, but his theory is both compatible with physio-

logical findings on the early stages of analysis in the visual system and also enables us to understand why the system is organised in the way that it is, given the requirements of the task it has to carry out.

The problem of three-dimensional vision

Virtually nothing is known about the physiological and anatomical arrangements that mediate the next stage of vision, that is the mechanisms that take as input the primal sketch containing information about the presence of lines and edges in the picture and compute a three-dimensional representation of the objects towards which the eyes are directed. In the 'sixties and early 'seventies, however, some programs²⁻⁴ were developed that successfully recovered a three-dimensional description of a scene from a line drawing. Most of this work was restricted to scenes containing only plane-faced polyhedra. Moreover, the 3-D descriptions formed were for the most part very impoverished. They usually

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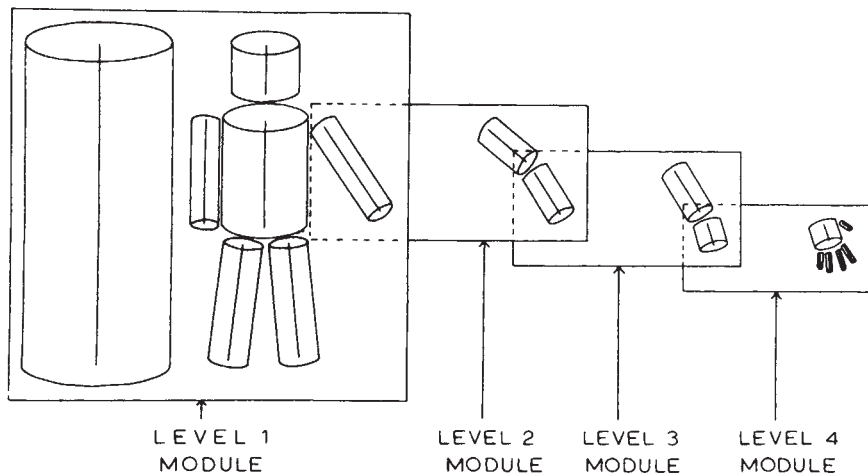


Fig. 1 Within each rectangle is shown on the left the 'model axis' for that level of description and on the right the way in which the model is broken up into component parts. The level 1 module represents the whole object, and the finer details of the arm, forearm and hand are represented respectively at levels 2, 3, and 4. At each level other than level 1, there would of course be many other modules representing for example the decomposition of the head, the nose and so on. Note that the body or component part represented at each level is decomposed into sub-components that are all of approximately the same size. (This figure is based on ref. 5.)

specified only the local relationships between pairs of surfaces in the scene: for example, two surfaces can be described as joining at a concave or convex edge or one surface may lie in front of another and partially obscure it. Even if additional information (such as the precise angle between two surfaces joined across an edge) is recovered and recorded, this kind of description is clumsy: considerable additional computation would be required to specify explicitly the different parts of an object and how they are related to one another. Moreover, anyone viewing an object has a strong impression of the volume of space it occupies and such information is not explicitly provided by a description that specifies only the relationships between neighbouring surfaces.

The construction by the brain of 3-D representations of objects serves two functions. First, in order to manipulate objects and to avoid bumping into them, organisms must be able to perceive the disposition of their surfaces in space. Second, in order to recognise an object and evaluate its significance for action, it is necessary to build a 3-D representation from the retinal image and then to match that description to a stored 3-D representation of the object. To facilitate the recognition process, it is likely that the representation constructed in immediate perception is similar in form to the stored representations with which it has to be matched in order for recognition to occur. In practice, the two processes—construction and matching—cannot be rigorously separated since, as will be

shown below, the construction of a perceptual representation may itself be guided by stored models of what is likely to be present in the environment.

The Marr and Nishihara model

Marr and Nishihara⁶ have recently made a fresh attack on the problem of how the brain represents information about 3-D objects: their proposals overcome some of the limitations of previous work. They lay down three criteria that such representations should satisfy in order to account for the efficiency with which the human visual system recognises 3-D objects.

Criterion (1) The representation should be easy to compute from the pictorial image.

Criterion (2) It should provide a unique description of any object which we are able to distinguish and the description should be canonical in the sense that the same object seen from any point of view will always yield the same unique description.

Criterion (3) Different representations should reflect the similarity between different objects whilst also preserving the differences: it is important to be able to recognise both that a shape is a man and that it is Jones or Smith.

One way of describing an object in three dimensions would be to compute the slope relative to the line of sight at each point on the visible surface of the object and the physical distance

between different points on its surface. Such a representation would be extremely cumbersome and would not satisfy criterion (2) since it would vary with changes in the observer's viewpoint. Marr and Nishihara argue for a volumetric description that is independent of viewpoint. To obtain such a description, some axis intrinsic to the object has to be selected to which the rest of the description can be referred: Marr and Nishihara term such an axis 'the model axis'. If the description of the same object seen from different points of view is to be canonical (criterion (2)), it is essential that when the same object is viewed on different occasions and from different viewpoints, the same model axis must be selected. For an elongated object, its longest dimension yields such a canonical model axis, and Marr and Nishihara only consider in detail objects of this sort. They suggest, however, that for some objects that are not elongated, like typewriters or armchairs, symmetry could be used to yield a canonical model axis. It is worth nothing parenthetically that for many objects commonly found in the same orientation, the visual system may select as a model axis the gravitational vertical.

Marr and Nishihara propose that objects are decomposed into their component parts. This proposal accords with our intuitions and has been previously made by others including myself⁷: some of my own experiments⁸ have indeed demonstrated that object recognition even in rats and goldfish is based on decomposing objects into their component parts. The originality of this aspect of Marr and Nishihara's work lies in their attempt to specify rigorously how such decomposition is effected, how the parts and their relationship to one another are described, and how the decomposition into parts can be used to facilitate the process of visual recognition.

In their theory the shape of each part is described as a generalised cone, that is, in terms of the surface swept out by a cross section moving along an axis and smoothly varying in shape and size. Binford⁹ was the first to suggest the use of generalised cones in the representation of 3-D objects, but Marr and Nishihara carry his suggestion much further by showing how it is possible to specify uniquely and simply the dispositions of the component parts of an object in terms of the relationships between the axes of the generalised cones by which they are represented. An important feature of their theory is that the descriptions are hierarchical: an object is first broken down into its main components and each of these is broken down into successively smaller sub-components.

Figure 1 illustrates such a representation. For an elongated form like that of a man, the longest axis is picked as the model axis and information is added about its length and the shape of the generalised cone that best approximates to the volume of space occupied by the whole shape. The object is next decomposed into its largest components and the component to which most other components are joined is then selected as 'the principal axis' for that stage of the description. Note that although in Fig. 1 the orientation of the principal axis of the whole form happens to be the same as that of the model axis, the two axes need not necessarily have the same orienta-

tion. Whereas the model axis depends on the direction of elongation of the whole body, the principal axis is the direction of elongation of the major component to which most other major components are joined. The orientation of the principal axis is specified in relation to the model axis: the angles and positions at which all other components join the principal axis are also recorded. The human form is most naturally decomposed initially into trunk, head, arms and legs with the trunk forming the principal axis. Where, as in the case of the arms and the trunk, the angle formed between one component axis and the principal axis is variable, information about the range of possible angles would also need to be stored. Figure 1 shows how in further modules the left arm can be decomposed into upper arm and forearm, with the forearm in turn decomposed into forearm and hand, and the hand into palm and fingers.

The resulting representation of a three-dimensional object is independent of viewpoint (criterion (2)), volumetric and modular. Marr and Nishihara call attention to three important advantages of modularity. First, it satisfies criterion (3): the similarity between objects can be extracted by examining the coarse representation of their major components, whilst small differences can be read off from modules that provide finer detail. Second, the model's modular construction makes it possible to effect changes in one module without disrupting others. Third, it facilitates recognition both of whole objects and of their parts.

Marr and Nishihara suggest a method of computing this type of representation from the visual image (criterion (1)). They consider primarily the construction of 3-D representations from silhouettes of objects: further details of the computational processes that effect the decomposition into parts and the assumptions underlying them can be found in Marr¹⁰. Figure 2 shows one way in which the main component axes may be recovered from the outlines of a donkey by segmenting the figure at its principal inflection points. Although this decomposition into component parts each with its own axis was achieved by a computer program, it is hard to evaluate how far the program would succeed with a range of other objects and how far it would succeed in producing the same segmentation with different viewpoints.

Even if the same segmentation is achieved, the initial description in terms of generalised cones and the relation between their axes will differ depending on viewpoint. In viewing real 3-D scenes, this problem can largely be overcome by taking into ac-

count cues (such as stereopsis and motion parallax) to the distance of different parts of the figure from the eye: such cues make it possible to compute the real length of the different axes and the real angle between one axis and another. Difficulties arise when the observer is presented with a two-dimensional picture of an object seen from a viewpoint that results in the foreshortening of the model axis. Marr and Nishihara consider a number of ways of dealing with this problem. If any part of an object can be recognised (for example, the foot of a horse), it would be possible to recover the stored representation of the whole object, and use that representation to guide the interpretation of the remainder of the picture. Moreover, the relative positions at which different parts of a body are joined to a principal axis are little affected by foreshortening and this information could again be used to retrieve the correct stored representation. Once the stored representation has been accessed, it is possible to estimate the orientation of the model axis with respect to the observer's viewpoint and to compute whether given this orientation the details in the image correspond to the model held in memory. Marr and Nishihara specify how this process may be carried out: it involves computing a 3-D representation of the object in viewer-centred coordinates, and adjusting the estimate of the orientation of the model axis by moving backwards and forwards between this representation and the representation stored in memory in object-centred coordinates.

Evaluation of the model

There are several difficulties in Marr and Nishihara's theory as developed so far, the first of which they note themselves. First, it is not clear that it would be possible to pick a model axis which remained the same for any given object regardless of viewpoint. As already noted, apart from the difficulties with objects that are not elongated, problems arise with elongated objects that are very foreshortened. This apparent drawback may in fact constitute one of the theory's strengths. Foreshortened pictures of common objects, for example, a pail or a telephone seen from directly above, are in fact very difficult to recognise (see Fig. 3). Second, it is not clear that it will always be possible to decompose the same object seen from different positions in the same way. Although at any level of modularity, the system is supposed to recover components of roughly the same size, decisions must be to some extent arbitrary. In Marr and Nishihara's own example (Fig. 1) an arm could be as readily decomposed into

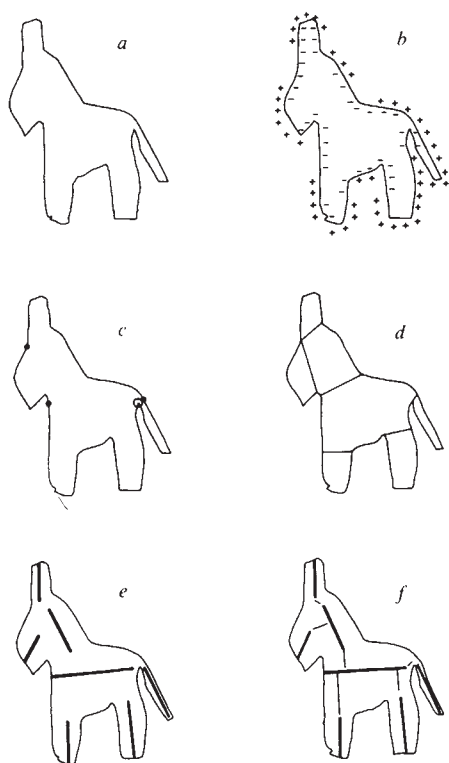


Fig. 2 The contours formed by the silhouette of a body composed of generalised cones can be used to locate the projections of its natural axes provided that they are not severely foreshortened. One method of decomposing the body and recovering the axes of the parts is shown in this example from a programme written by P. Vatan. The initial outline (a) was obtained by applying local grouping processes to the 'primal sketch' of an image of a toy donkey. This outline was then smoothed and divided into convex (pluses) and concave sections (minuses) (b). Next, strong segmentation points, like the deep concavity circled in (c), are identified and a set of heuristic rules are used to connect them with other points on the contour to produce the segmentation shown in (d). The component axes shown in (e) were then derived. The thin lines in (f) indicate the junctions of the head, leg and tail components to the torso axis, and of the snout and ear components to the head axis (figure and legend based on ref. 5).

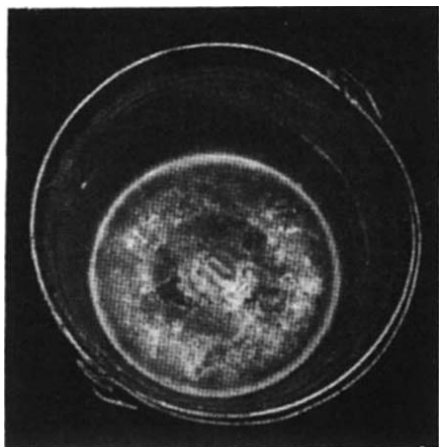


Fig. 3

upper-arm, forearm and hand as into upper-arm and forearm (with the latter being decomposed in a further module into forearm proper and hand). The problem of decomposition into components of approximately the same size becomes particularly acute if some of the components are very foreshortened. Failure to decompose an object in the same way would make it difficult to match the initial representation of the object to the representation stored in memory. Third, the model seems in some ways to exceed the capacities of human vision. Rock¹¹ and others have found that elongated 2-D shapes are not readily recognisable when rotated in the picture plane unless the observer has had experience of seeing them in several orientations.

The proposed model does, however, have many new and useful features. As stressed above, the theory facilitates the computational processes underlying object recognition. It is virtually the first attempt to specify a general theory of the form in which visual representations of 3-D objects are stored. It is an advance on most previous theories in that it handles objects with curved surfaces and silhouettes of objects: although there is less difficulty in constructing unambiguous representations when viewing the real world directly, people can form 3-D representations from silhouettes and it is necessary to account for this capacity.

Moreover, in addition to its computational power, the model has in my opinion considerable plausibility on psychological grounds. First, in looking at an object we are simultaneously aware both of its visible structure with respect to our viewpoint and also of the volume of space it occupies: Marr and Nishihara compute both of these representations and give good reasons why it is necessary to do so. They show

how recognition can be facilitated by adjusting a representation in viewer-centred coordinates by fitting it by successive approximations to a stored model which is independent of viewpoint. It might be added that no matter how much constructing a description that is independent of viewpoint facilitates recognition, it would still be necessary to build a description in terms of viewer-centred coordinates in order to manipulate objects. Second, their model explains the difficulty experienced in recognising pictures of foreshortened objects. Third, although Rock¹¹ found that recognition of elongated objects rotated in the fronto-parallel plane was difficult, they were easier to recognise under rotation than objects that were not elongated. Fourth, as Marr and Nishihara note, there is evidence that people sometimes refer the description of the parts of an object to its longest axis. One instance, to which Mach¹² was the first to draw attention, is shown in Fig. 4. The single large diamond is seen as a diamond—its description is referred to its vertical axis; the diamonds forming a diagonal line are, however, usually seen as squares—their description is referred to the orientation of the model axis defined by the direction of elongation of the whole line. Finally, the theory captures our intuition that in viewing an object we see it (and verbally describe it) as composed of discrete parts each occupying a volume of space. Moreover, if it is assumed that it is possible to be conscious of the details of only one of the modules at a time, the theory satisfies our intuition that we cannot simultaneously form an image of the general shape of a man and of the details of the shape of his left ear.

The problem to which Marr and Nishihara address themselves is how

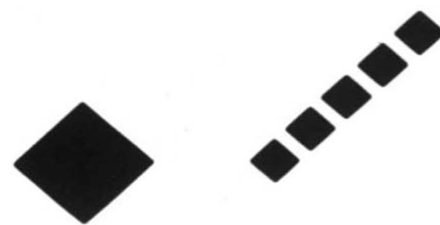


Fig. 4 The single diamond is seen as a diamond because its orientation is perceived with respect to the vertical; but the diamonds in the oblique line tend to be seen as squares because their perceived orientation is determined by their relationship to the diagonal line of which each is a part.

we recognise a given object as a member of a class of objects all of which have the same basic physical shape. It is worth noting that there is a further and even deeper problem of visual perception to which neither they nor anyone else have provided any solution. This problem is how we recognise the category to which a new object belongs when the new example has little or no similarity of shape to examples previously encountered, but does have the same function. Chairs come in a great variety of shapes and sizes and all they have in common is that they are man-made objects having a surface suitable to receive the weight of the human bottom and another fitted to support the human back. Nevertheless, we have little difficulty in recognising, even when first encountered, bizarre new chairs thought up by futuristic designers: in order to do so, we must integrate our knowledge of the function of chairs with the 3-D description formed of the new example.

It has of course been impossible to present here more than an outline of Marr and Nishihara's paper which contains many subtle points of detail. As with all Marr's work, the decisions he has taken in theory construction are always motivated: there is none of the 'I put that in because it seems to work, goodness knows why' attitude that characterises the *ad hocery* of some practitioners of artificial intelligence. Marr and Nishihara's paper provides a starting point, not a finished theory. Nonetheless, it contains some brilliant insights into a deep, complex, and much worked over problem, and it is possible that in a few years' time it will be regarded as a classic. □

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The Ice-age Amazon

from Paul Colinvaux

A MAP has been published by Ab'Saber (*Paleoclimas* 3, 1; 1977) which shows the Amazon basin to have been largely without closed forest during the last ice age. Wet forest was restricted to the eastern flanks of the Andes, to the mountains of Guiana in the north, along the coastal escarpment of eastern Brazil, up which there now climbs the spectacular highway linking São Paulo with its port of Santos, and in scattered patches along the Amazon river system. This map is the most definite statement yet of a growing consensus that equatorial South America has been subjected to major climatic change. The importance of this to both climate dynamics and ecological theory is very great.

The most persuasive direct evidence for environmental change in the Amazon basin is the existence of dissected and gullied land surfaces now covered by closed forest. These surfaces were revealed by side-scan radar from aircraft in surveys made by the Brazilian government. Complete maps from the side-scan survey do not seem to have been published but I was assured in Brazilia that the coverage is extensive. Both Tricart (*Rev. Geomorph. Dynamique* 23, 145; 1975) and Bigarella & Becker (*Bol. Paranaense Geosci.* 33, 171; 1975) have given accounts of the fossil land forms, showing that at times in the Pleistocene wide stretches of the Amazon cannot have supported closed forest. Ab'Saber's map is a further compilation of these data, coupled with the results of many years of geological and soil survey. Dating of the dissected surface is still weak but Bigarella & Becker show that outside the Amazon basin near São Paulo, the last episode preceded a radiocarbon date of 10,000 b.p., thus allowing the event to be synchronous with northern glaciations.

The ice age climate of equatorial America is also charted in the high Andes and in the Galapagos Islands. Andean lakes from Titicaca in Peru to Bogota in Colombia have retreated from shorelines of much larger lakes in the recent geological past. The shorelines themselves have not yet yielded radiocarbon ages of their occupancy, but a powerful argument suggesting that the larger lakes were associated with glaciations comes from studies of end moraines abandoned by the Quelccaya ice-cap in Peru. The Quelccaya is the world's third ice cap, perched improbably on the mountains within 14° of the equator. Continuing mass-balance studies of the ice-cap by

Thompson *et al.* (*Science* 203, 1240; 1979) show that precipitation must be heavy to offset the high annual ablation and that an ice-cap can only be maintained when clouds shield the ice for many months each year. Extension of the ice-cap in the past, therefore, implies increased precipitation and cloud, conditions which would favour enlargement of the lakes and suggesting that their growth was synchronous with Andean glaciations.

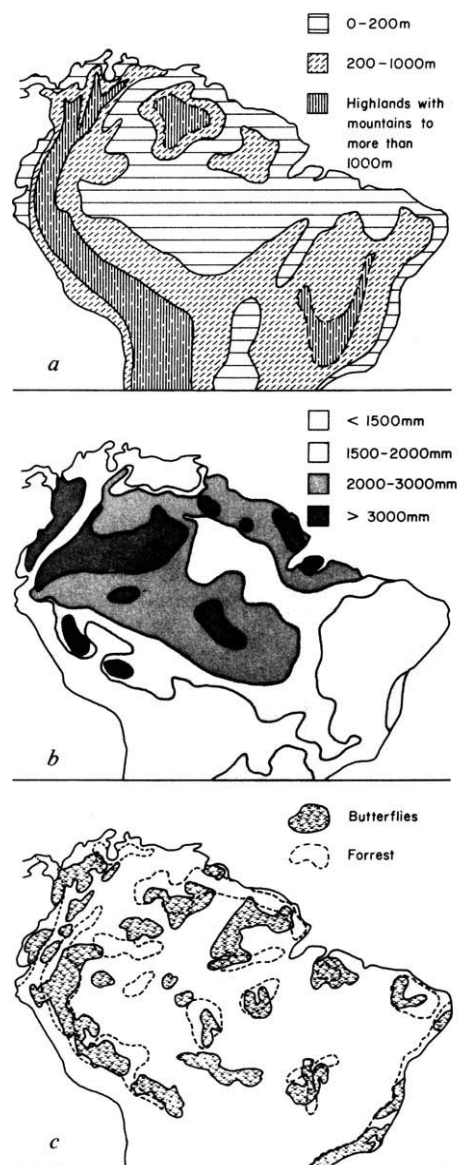
Finally there is well-dated sediment sequence from the Galapagos Islands which shows that the moist highlands, which are now shrouded by stratus cloud and fog-drip, were dry from 10,000 b.p. to a time beyond the reach of radiocarbon dating (see *Nature* 240, 17; 1972). The Galapagos record has the securest dating, encouraging the impression that climate along an equatorial strip across the continent suffered many changes during the last glaciation of the northern hemisphere. There was increased dryness at the Galapagos to the west, increased wetness in part of the high Andes, and a lack of forest cover in the Amazon basin (suggesting seasonal rain and drought) all the way across the continent to the edge of the coastal escarpment in the east. A possible implication for climate dynamics is that the Intertropical Convergence Zone (ITCZ) was largely obliterated in glacial times, as it is in Africa. At least there seems good reason for thinking that the zone was neither to the south nor to the north of its present position (see *Nature* 240, 17; 1972; 245, 91; 1973). But the more exciting implications are for ecological theory. A review of the latest data on the distribution of modern taxa by Simpson and Haffer (*Ann. Rev. Ecol. Syst.* 9, 497; 1978) is thus of great interest.

There are many Amazon data about butterflies compiled from the very large collections which have been continually growing since the first of their kind by Waterton and Wallace in the last century. Bird data are also more complete than one might expect and have been supplemented by less complete collections of lizards and some plants by Simpson and Haffer. The data show that there are many, highly local, definite, disjunct distributions. What is more, the disjunctions can be shown, particularly with the butterfly data, to be bordered by zones where arrays of hybrids are normal. Turner (*Evolution* 25, 471; 1971) compared the marking of modern hybrid butterflies with those illustrated in Dutch books of the 18th century and showed that the same hybrids have been produced for 200 yr, or 2,000 generations, making plausible the postulate that the present patterns of distribution are not changing rapidly. As these patterns of distri-

bution define patches within continuous rain forest today, Simpson and Haffer seek their origin in the past. They postulate that each centre of distribution represents a forest refuge in the more seasonal or drier climates of the last ice age. In a footnote to their paper they suggest that the forest refuges shown on the Ab'Saber map can be reconciled with these modern centres of distribution.

The figure shows sketch-maps of the region for modern topography, modern rainfall, and a summary of dispersal data for butterflies. Very approximate boundaries for the ice-age forests of Ab'Saber are added to the butterfly map.

There is a strong and obvious synchrony between many butterfly distributions and the postulated forest



a, Modern topography; b, modern rainfall; c, distributions of modern butterfly populations (shaded) and ice age forest refuges estimated from fossil land forms (dashed lines).

refuges but these tend to be along the mountains or escarpments that ring the Amazon Basin. On the lowlands themselves correlations are scarcely conclusive. It is probable that traces of the ice age Amazon are indeed stamped on modern distributions as Simpson and Haffer suggest, yet it may still not be the most parsimonious of hypotheses to say that the past has been decisive. The way must be open to ecological hypotheses explaining the patterns of distribution from modern rainfall, modern topography and the channels of modern rivers. These hypotheses may well be the more parsimonious, at least without detailed mapping of the ancient vegetation by such means as pollen analysis, something on which no start has yet been made.

Nevertheless, it is clear that all hypotheses of the high diversity of tropical biota must now reckon with the fact that the equatorial lowlands are not immune to climatic change, being in

fact subject to environments with each northern glaciation sufficiently different to replace one biome with another. One of the most attractive of the hypotheses for high diversity has been that of Sanders (*Amer. Nat.* **102**, 243; 1968) who showed that long physical stability on ocean floors is associated with high diversity, in spite of low productivity. The suggestion that high stability in equatorial latitudes accounted for the high species list in a similar way has been attractive to many of us. We now have to modify our views. Possibly, the important part of the stability of tropical systems is still that areas must always have persisted in which seasons were minimal, because without them the biota of the wet tropics as we know them cannot have survived. The areas may have moved around a bit, but they had to be there. In these places we can still postulate lower extinction rates, higher speciation rates, or more niche-overlap as our fancy dictates. □

Difficulties in the definition of new hominid species

from our Palaeoanthropology correspondent

As a group, palaeontologists have a notable tendency to propose new names for the fossils they find. Whether from scientific profundity or other motives, new binomials seem to appear with an almost greater frequency than the fossils themselves. Historically, the same has been true of human palaeontologists; proposals of five or six different binomials for the same fossil hominid are not unknown. Paradoxically, however, the enormous number of new hominid fossils that have appeared in this decade has generated very little taxonomic activity. One exception, 'Homo ergaster', has been permitted to enter an unmourned and well-deserved obscurity.

Now, Don Johanson and Tim White have proposed a new species for the Plio-Pleistocene hominid genus, *Australopithecus* (*Kirtlandia* **28**, 1, 1978; *Science* **203**, 321; 1979). The species, *A. afarensis*, is based on specimens found at Laetoli in Tanzania and in the Hadar area of Ethiopia; they appear to date between 2.9 and 3.8 million years ago. While the majority of this material has been recovered very recently, the 'Garusi' maxilla was recovered at Laetoli in 1939 by Kohl-Larsen.

A workable definition of a fossil species continues to elude palaeontologists. While recognising, as do neontologists, that genetic isolation is the prime criterion for species formation,

the way such isolation can be recognised in the fossil record is unclear. Most definitions centre on the demonstration of morphological distinctiveness; a fossil species should therefore have a clearly defined and distinguishing set of morphological characters. This is apparently the sort of definition that Johanson and White have worked with. However, their success in demonstrating the morphological distinctiveness of *A. afarensis* is by no means clear. Their difficulty, and the difficulty of those who try to evaluate their classification, lies equally in the incompleteness of the fossil specimens and in the wide morphological variability of early (as well as recent) human populations. Moreover, the type specimen of the most appropriate comparative taxon, *Australopithecus africanus*, is a juvenile. The fact that this specimen, the Taung child, has only the first molars of its permanent dentition in place has long caused taxonomic difficulty. For this reason, the gracile australopithecines from Sterkfontein, in South Africa, are usually regarded as representatives of this species. White and Johanson have also included the australopithecines from Makapan, along with those from Sterkfontein, in their comparative group. However, the gracile status of the Makapan hominids is not certain. They do demonstrate several cranial and dental characters more often found in the heavily built

'robust' australopithecines and have, in fact, been placed with that group by some workers. A further problem with White and Johanson's comparative group is that they appear to have included dental measurements from Taung for teeth (4th premolars and 3rd molars) which the child did not possess (*Science op. cit.*).

White and Johanson recognise that it is the total pattern of characters which must define a taxon and set it apart from other species. While bearing this in mind, it must be pointed out that very few characters of *A. afarensis* are not also found in *A. africanus*. For example, important distinguishing features of the new species are said to occur in the anterior premolars of the mandible. In *A. afarensis* this tooth has a large outer cusp and a very small inner cusp; the long axis of the tooth is rotated 45 degrees or more to the long axis of the tooth row. Yet a similar pattern of morphology is seen in the Miocene genus *Ramapithecus* (regarded by some workers as an ancestral hominid) and is also found in the Sterkfontein specimens (Robinson *Transvaal Museum Memoirs* No. 9, 72; 1956). Similarly, the mandibular canines of *A. afarensis* are said to be large, yet in both length and breadth means this tooth is larger in the Sterkfontein sample (Robinson *op. cit.*). Other features, such as three-rooted upper premolars and an increase in molar size from front to back can also be duplicated in the South African sample. Morphological congruence, rather than distinctiveness, can also be demonstrated in the post-cranial remains. One of the wrist bones, the capitate, of *A. afarensis* is described as "waisted" and the third metacarpal, with which it articulates, is said to lack a styloid process. A capitate from Sterkfontein has a similarly constricted shape on its lateral surface (Le Gros Clark *J. Anat.* **81**, 300; 1947; Lewis, *J. Hum. Evol.* **2**, 1; 1973). Moreover, while a third metacarpal is not known from this site, the articular area on the Sterkfontein capitate may indicate that the styloid process of the metacarpal was also missing (Le Gros Clark *op. cit.*). Finally, the innominate of one member of *A. afarensis* (AL 288-1 or 'Lucy') is extremely similar in both size and morphology to one from Sterkfontein.

It is also true, however, that the *A. afarensis* specimens do show characters not known to be represented in *A. africanus*. For example, the upper central incisors are said to be very broad in the earlier group and the lateral incisors relatively small. However, the incisors are very poorly known for both *A. afarensis* and *A. africanus*. While the known central

incisors are, indeed, very large in *A. afarensis* they are known from only two individuals. The comparative sample from Sterkfontein consists of a single individual. The very limited sample of lateral incisors from Sterkfontein ($n=2$ individuals) shows this tooth to be smaller than the very "reduced" *A. afarensis* tooth. In the *A. afarensis* maxilla the canine characteristically projects below the level of the other teeth and a diastema is present in the tooth row. Although this is often regarded as a primitive feature, at least one *Homo erectus* individual, from Java, also shows a similar pattern. It should be pointed out that in the original reports on the Hadar material, specimens showing these features were placed not in a primitive hominid group as they now are, but in the more advanced genus, *Homo*.

Thus, there are very few features said to be characteristic of *A. afarensis* which are, in fact, distinctive of that group. Perhaps a major feature of the specimens now grouped into *A. afarensis* is the amount of variability present. This variability originally led to the informal proposal that as many as three taxa were present at Hadar. Therefore, while the original reports maximised the taxonomic importance of this variability these current reports have, in effect, minimised it to the level of sexual dimorphism. Undoubtedly, considerable sexual dimorphism was present in hominids of the Plio-Pleistocene but can such dimorphism explain all the variability present at Hadar and Laetoli? Perhaps, but both the recovery of additional material from this important time period as well as more careful comparative studies of existing specimens are required before the validity of *A. afarensis* can be affirmed. □

Death of a schistosome

from F. E. G. Cox

Schistosoma mansoni in laboratory rodents is a useful model for the study of schistosomiasis and much of what we know about the biology of the parasites that cause the human disease has been derived from studies on rats and mice. The infection in a mammal begins when free-swimming cercariae, released from a snail intermediate host, attach to and penetrate the skin. While this is happening, the cercariae rapidly transform into schistosomula by discarding the glycocalyx required during their sojourn in freshwater and reveal-

ing the characteristic double bilayer outer membrane. Each schistosomulum then makes its way to the blood vessels, stimulating an immune response but avoiding its consequences by acquiring red cell antigens that render it immunologically invisible. However, subsequent schistosomula that have not acquired host antigens are fully susceptible to attack from the immune system and thus superinfection is effectively prevented. The schistosomulum is the obvious weak link against which any immunological attack must be directed and the development of a vaccine against schistosomiasis will depend to a large extent on our understanding of what happens to the schistosomulum during the first few hours in its host.

The immune responses against schistosomula in previously sensitised hosts are extremely rapid and effective and several effector mechanisms have been postulated including antibody and complement and complement activated through the alternative pathway. Recently, various cells have also been found to be involved, in particular macrophages in the presence of IgE (A. Capron *et al.* *Nature* **253**, 474; 1975), eosinophils in the presence of IgG (A. Capron *et al.* *Amer. J. trop. Med. Hyg.* **26**, 39; 1977; Mackenzie *et al.* *Clin. exp. Immun.* **30**, 97; 1977) and eosinophils in the presence of complement (Ramalho-Pinto, McLaren & Smithers *J. exp. Med.* **147**, 147; 1978). It is known that eosinophils adhere to a schistosomulum through either Fc receptors or complement receptors and, in *in vitro* systems, empty their contents on to the surface of the worm (McLaren, Mackenzie & Ramalho-Pinto *Clin. exp. Immun.* **30**, 105; 1977). Until now, however, the evidence that eosinophils actually kill the schistosomulum has been circumstantial: labelled chromium release, for example, is increased but no ultrastructural damage has been detected. It has now been conclusively shown by the group of workers at Mill Hill that the schistosomulum is damaged and killed (McLaren, Ramalho-Pinto & Smithers *Parasitology* **77**, 313; 1978).

The death of the schistosomulum begins when eosinophils attach to its surface, flatten out and release their contents causing a small lesion in the tegument through which the cells migrate into the interstitial tissue of the worm. The tegument is prised off and the worm dies. The actual method of attachment of the eosinophils to the surface of the worm is important as is the ratio of cells to schistosomula. 3,000–5,000 eosinophils per schistosomulum are necessary (3,000 were used

in the previous studies) and attachment through complement receptors results in 100% killing in 20 h whereas attachment through Fc receptors results in little damage within 20 h and, in some worms, no damage at all.

The eosinophil product that actually causes damage to the schistosomulum seems to be the major basic protein (MBP) from the eosinophil crystalloid core (Butterworth *et al.* *J. Immun.* **122**, 221; 1979). The destructive effects of MBP are nonspecific but schistosomulum killing is effectively specific because of the tight binding of eosinophils to antibody-coated worms and the release of MBP into a small space between the eosinophil and the schistosome surface. The action is therefore localised and no bystander host cell damage occurs.

There is now abundant evidence that eosinophils in conjunction with either IgG or complement can kill schistosomula but the whole story may be more complicated than this. Monique Capron and her colleagues at Lille have observed that both eosinophils and mast cells adhere to schistosomula and have found that, in the presence of IgG and with 5,000–10,000 effector cells per worm, the proportion of mast cells to eosinophils is a very important factor in killing (M. Capron *et al.* *Europ. J. Immun.* **8**, 127; 1978). The cytotoxic effects decrease as mast cells are depleted and increase with mast cell enrichment. Ultrastructural studies show that little damage is done to the tegument in the first 3 h but that it is very vacuolated after 20 h, findings comparable with those of the Mill Hill group. In a further series of experiments (M. Capron *et al.* *J. Immun.* **121**, 2518; 1978) it has been shown that eosinophil-dependent cytotoxicity proceeds in three steps, the activation of the mast cells, the release by these cells of a soluble mediator that acts as a signal to the eosinophils and the reception of this signal by the eosinophils. Activation of the mast cells depends on IgG binding to the schistosomulum, the IgG involved being IgG2a, the second anaphylactic antibody in rats. This antibody also seems to be involved directly in eosinophil-mediated cytotoxicity. As both mast cells and eosinophils possess receptors for IgE and complement there may be several patterns of eosinophil activation in schistosomiasis and these may act separately or together. Whether the eosinophil-complement mechanism also involves mast cells remains to be determined, for mast cells also adhere to schistosomula through complement receptors (Sher & McIntyre *J. Immun.* **119**, 722; 1977).

An understanding of the role of eosinophils in immunity to helminth infections is likely to become in-

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creasingly important as the attachment of these cells and the release of their contents have also been seen with the adult nematodes, *Nippostrongylus brasiliensis* and *Trichinella spiralis* (McLaren, Mackenzie & Ramalho-Pinto *Clin. exp. Immun.* **30**, 105; 1977) and larval *T. spiralis* (Kazura & Grove *Nature* **274**, 588; 1978). The finding that the nematode *Ascaris suum* contains a substance that is chemotactic for eosinophils and neutrophils (Tanaka, Baba & Torisu *J. Immun.* **122**, 302; 1979) may mean that certain worms are actually instrumental in their own destruction. Immunity to helminths is complex and there are unlikely to be any simple explanations of all the facts observed so the vast amount of fundamental research being carried out is easy to justify in terms of its possible importance to our understanding of human and animal diseases.

Growing embryos *in vitro*

from Gillian Morriss

THE once inaccessible postimplantation mammalian embryo can today be grown in transparent vessels in the laboratory for various periods between implantation and an advanced stage of organogenesis. This is largely due to the work of New and his collaborators in Cambridge, and (the earliest stages) Hsu and colleagues in Baltimore (reviewed by New, *Biol. Rev.* **53**, 81; 1978). Mammalian embryologists have been slow to exploit the opportunities of whole embryo culture, but recent progress in the culture technique represents a significant increase in its potential, since the *in vitro* requirements of postimplantation mouse embryos have now been defined.

Pre and peri-implantation culture of mouse embryos are now routine procedures, whereas for postimplantation stages the techniques were previously well worked out only for the rat. Rat embryo culture is an important technique for the investigation of environmental factors such as energy supply, gas phase constituents, and chemical teratogens. For specific chemical interference with intra or extracellular components such as basement membrane collagen, glycosaminoglycans and microfilaments, it is immaterial which mammalian species is used. However, for the investigation of abnormal development which is genetic in origin,

mouse mutants provide a range of abnormalities unrivalled by any other experimental species. These mutants represent an important potential source of information on normal and abnormal mechanisms of mammalian morphogenesis. The onset of abnormal development of several of them occurs during the early postimplantation period (primitive streak stage to mid-organogenesis), and *in vitro* studies will make an important contribution to elucidation of the mechanisms involved.

Y.-C. Hsu (*Dev. Biol.* **68**, 453; 1979) has achieved a significant improvement in the proportion of preimplantation stage mouse embryos which attach *in vitro*, develop into 'egg cylinders' and subsequently reach the 5-10-somite stage. The technical alterations were simple: blastocysts were cultured singly in 2 ml medium in 35 mm culture dishes, and 2 days after attachment (early 'egg cylinder' stage) the serum component in the medium was changed from 20% fetal calf serum to 20% human cord serum (the synthetic component was CRML 1066 throughout). Twelve out of twenty embryos developed to the 5 to 10 somite stage, after which they began to degenerate. It is important to note that the photographs of these 'successful' embryos closely resemble rat embryos grown at this stage in dish cultures: the spinal neural tube closes, but the cranial neural folds fail to change from their early convex shape to the concave shape which precedes closure of the brain tube.

From the primitive streak stage onwards, rat embryos undergo normal morphogenesis only when the culture vessel and/or the culture medium is continuously moving. T. W. Sadler (*J. Embryol. exp. Morph.* **49**, 17; 1979) has modified this technique for the culture of mouse embryos from the 2-4-somite stage for up to 48 h. During this period mouse embryos require a greater volume of gas phase than rat embryos; however, their liquid requirements are apparently identical, with rat serum supporting normal development. Successful culture of younger mouse embryos, from the primitive streak stage for 48 h, has been achieved by M. Snow and P. P. L. Tam (personal communication), using 30% newborn calf serum in Dulbecco's modified Eagle's medium supplemented with glucose (4.5 g l⁻¹).

Meanwhile, the better-established techniques of rat embryo culture are yielding information on the metabolic requirements of the early embryo, and on some aspects of the relationship between these and morphogenesis. Klein *et al.* (*J. exp. Zool.* **203**, 313; 1978) have improved development of 10-day embryos in small quantities of serum (four embryos per 2 ml) by changing the serum after 24 h. The im-

provement was related to depletion of a protein (or proteins) of approximately 125,000 molecular weight; this protein may be identical to the α 2-globulin of 126,000 molecular weight, identified by Hoch and McVey (*J. biol. Chem.* **252**, 1881; 1977) as one of the two major human serum DNA-binding proteins.

Energy production of 10-d embryos is primarily through anaerobic glycolysis (Tanimura & Shepard *Proc. Soc. exp. Med. Biol.* **135**, 51; 1970); subsequently (day 10 to day 12) oxygen dependence progressively increases, as the yolk sac and then the chorioallantoic placental circulations develop. Not surprisingly, these observations are matched by the oxygen requirements of cultured embryos (reviewed by New *op. cit.*). Robkin and Cockcroft (*Teratology* **18**, 337; 1978) have compared the effects of oxygen deprivation and carbon monoxide elevation on cultured 11-12-d embryos. Decreased availability of oxygen produced a greater response (depression of metabolism and growth rate) than elevated carbon monoxide, and the authors suggest that at this stage of development oxygen transport is primarily through solution rather than in combination with haemoglobin.

Our understanding of the mechanisms involved in controlling most aspects of normal and abnormal organogenesis has progressed very little compared with progress in other areas of developmental biology during the past decade or so. It is to be hoped that the recent improvements in techniques of mammalian embryo culture will soon be fully utilised in pursuit of such studies. □



A hundred years ago

ON Tuesday morning, in the presence of a small number of his sorrowing friends, the remains of the late Prof. W. K. Clifford were placed in their last resting-place in Highgate Cemetery.

THE following grants have lately been made from the Research Fund of the Chemical Society: —10l. to Dr. C. A. Burghardt for an investigation into the constitution of topaz; 20l. to Mr. Francis Jones for the investigation of boron hydride; 15l. to Mr. F. D. Brown for the study of the theory of fractional distillation; 30l. to Dr. Dupré for the estimation of organic carbon in air; and 15l. to Prof. T. E. Thorpe for the investigation of albitene, the hydrocarbon of nut-pine. From *Nature* **19**, 27 March, 491; 1879.

review article

The new frontier of particle physics

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Quarks and leptons have emerged as elementary constituents of matter. To these may perhaps be added the field particles, or mediating agents, of a unified theory of the weak and electromagnetic forces and also those of a new theory of the strong (nuclear) force. This article briefly outlines some of the main items of evidence for this new picture and indicates a few of the many crucial questions to be answered.

THE first lepton to be discovered was the electron, by J. J. Thompson in 1897. The leptons are a family of particles with spin 1/2 (in units of $\hbar$) and a membership summarised in Table 1 which includes two recent additions.

The charged leptons participate in both the electromagnetic and the weak interaction (responsible for processes such as radioactive β -decay) while the neutrinos, with zero electric charge, are influenced only by the weak force.

Since its discovery 40 years ago, the muon has remained one of the great enigmas of particle physics; it differs from the electron only in mass, has equal strength couplings to the electromagnetic and weak forces and shows no sign of additional modes of interaction which might account for this mass difference. Moreover, in 1961 the electron and muon were found to be associated with different neutrinos, ν_e and ν_μ . Another lepton pair has now been added to the list; with the matching expansion in the numbers of types of quarks this multiplicity of forms is one of the most tantalising aspects of particle physics at present.

The annihilation of high energy electrons (e^-) and positrons (e^+) brought together in the head-on collision of oppositely circulating beams of e^- and e^+ can lead to the creation of charged lepton pairs or numbers of hadrons (particles which participate in the strong interaction as well as in the weak and electromagnetic interactions). The τ -lepton was discovered in 1975 by Perl *et al.*¹ using the e^+e^- machine SPEAR built at the SLAC laboratory. Its existence has been confirmed by experiments² at the similar machine, DORIS, at the DESY Laboratory, Hamburg. It is almost certainly associated with a new neutrino, ν_τ , and although the mass limits on ν_τ are still high it is for the moment assumed, like ν_e and ν_μ , to have zero mass.

The magnetic moments of the electron and muon have been measured to remarkable precision in two experiments of great

technical ingenuity and scientific elegance. The work on the electron, by Dehmelt *et al.*^{3,4} at Seattle has reached an accuracy of 1 part in 10^{10} and at CERN the group led by Picasso⁵ has achieved 1 part in 10^8 for this static property of the unstable muon. These experiments test the validity of quantum electrodynamics (QED), the quantised electromagnetic theory; they also probe the structure of the particles and the possibility of couplings to additional fields of force. Dirac's theory for point-like particles of spin 1/2 predicts a magnetic moment of $\mu_D = e\hbar/2mc$ (m is the particle mass), however, the measured values are very slightly higher (by about 1 part in 10^3) and the difference is called the anomalous magnetic moment. In QED the anomalous moment is due to corrections to μ_D , called radiative corrections, which arise from interactions of the charge with the electromagnetic field; these can be illustrated by the

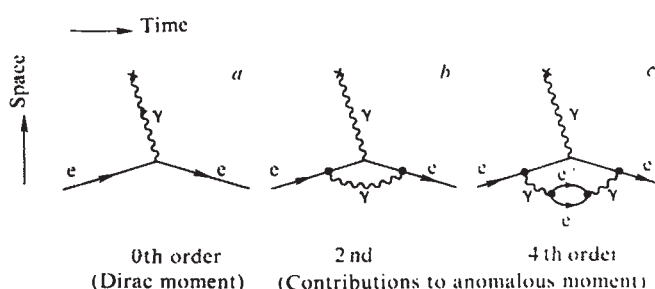


Fig. 1 Feynman diagrams showing contributions to the magnetic moment of the electron.

Feynman diagrams of Fig. 1. (Feynman diagrams illustrate details of the interactions between particles and fields by showing their evolution in space and time; with a set of rules given by Feynman they provide a prescription for calculating transition rates. One space dimension is shown and time 'flows' from left to right.)

The lowest order diagram corresponds to interaction with a virtual photon, mediator of the external electromagnetic field, and gives the Dirac moment, μ_D ; the radiative corrections involve emission and re-absorption of virtual photons, including in higher orders the transient presence of virtual charged lepton pairs. This 'structure' associated with the field is the source of the anomalous moment. The calculations have been carried out to sixth order (96 diagrams contribute) and the excellent agreement between theory and experiment^{6,7} represents a remarkable success for QED. In addition the experiments lead to the following conclusions about the electron and muon: (1)

Table 1 The leptons (and anti-leptons)

Name		Charge*	Mass (MeV)†	Lifetime
Electron	$e^-(e^+)$	-1(+1)	0.51	Stable
Electron-neutrino	$\nu_e(\bar{\nu}_e)$	0	$0(<4 \times 10^{-5})$	Stable
Muon	$\mu^-(\mu^+)$	-1(+1)	105.7	2.2×10^{-6} s
Muon-neutrino	$\nu_\mu(\bar{\nu}_\mu)$	0	$0(<0.5)$	Stable
τ -lepton	$\tau^-(\tau^+)$	-1(+1)	1807	$<3 \times 10^{-12}$ s
τ -neutrino	$\nu_\tau(\bar{\nu}_\tau)$	0	$0(<300)$	Stable(?)

*In units of the electron charge, e .

†The proton mass in these units is 938.3. The neutrinos are assumed to have zero mass; the present experimental limits are given in parentheses.

there is no evidence of a new coupling sufficient to account for the heavier mass of the muon; (2) the electron and muon behave like point-like, structureless particles at least down to distances approaching 10^{-15} cm.

The quarks

Leptons can be created and their properties measured to very high precision in the laboratory. In contrast all we know about quarks has to be inferred from indirect information like the patterns of hadron spectroscopy, the products of e^-e^+ annihilation, the scattering of leptons probing the nucleon and similar processes. This is a severe technical difficulty, and the failure to establish the existence of free quarks has cast doubt on this concept. These difficulties make it worth summarising some of the evidence which has led to the present common acceptance of the quarks as constituents of the hadrons.

The quark proposal^{8,9}, made by Gell-Mann and independently by Zweig in 1964, was the culmination of a successful attempt to explain the numerous spectrum of hadronic states (more than 200 are known). There are two main classes of hadrons: the baryons have half-integer spin and include the proton and neutron (nucleon); the mesons have integer spin. (All particles of half-integer spin are called fermions and those of integer spin are called bosons.)

When the quark model was proposed only three components were required to 'build' all the known hadronic states; these were the u, d and s quarks listed in Table 2. The u and d quarks form an isospin doublet (like the proton and neutron), while the s quark carries the attribute of strangeness, a hadronic property discovered in the late 1940s. The different quark types have been given the collective name 'flavours'. Quarks not only have the exotic property of fractional electric charge, which should make them easily recognisable if freed from hadronic confinement, but also baryon-number (B) of $1/3$ ($-1/3$ for $\bar{q}$). Baryon-number $+1$ is assigned to all baryons (-1 to anti-baryons) and 0 to all mesons; it is a quantity believed to be strictly conserved by all interactions, leading to the apparently absolute stability of the lightest baryon, the proton.

The baryons are formed from three quarks ($3q$) and the mesons from quark-antiquark pairs ($q\bar{q}$); examples are: proton (uud), neutron (udd), π^+ -meson ($u\bar{d}$) and K^+ -meson ($u\bar{s}$). The ($3q$) and ($q\bar{q}$) recipe assembles multiplets of states, all with the same spin and parity; the multiplets formed from the u, d and s quarks correspond to those representations of the unitary symmetry group $SU(3)$ which are just the ones needed to accommodate the states observed (at least up to a mass of 3 GeV). The quark triplet corresponds to the simplest (non-trivial) representation of $SU(3)$. Baryons ($3q$) form multiplets of dimension (number of members) (1), (8) and (10) while the mesons ($q\bar{q}$) occur in (1) and (8).

Invariance to transformations of the $SU(3)$ group would imply equal mass for all the members of a multiplet. However, this symmetry is broken to the extent that mass differences occur; one may accommodate these by making the s-quark ~ 150 MeV heavier than the u and d quarks. The masses and magnetic moments of the low mass hadrons can be understood if the quarks have spin $1/2$, have Dirac-like moments and if the effective masses of the u and d quarks are ~ 350 MeV. (But these should not be taken as the masses free quarks might have—if they exist.)

Table 2 Quarks and anti-quarks

Name (Flavour)	Charges			
	q	$\bar{q}$		
	+2/3	-1/3	-2/3	+1/3
Up	u	$\bar{u}$		
Down		d	$\bar{d}$	
Charm	c	$\bar{c}$		
Strange		s	$\bar{s}$	
[Top?]	[t]		$[\bar{t}]$	
Bottom		b	$\bar{b}$	

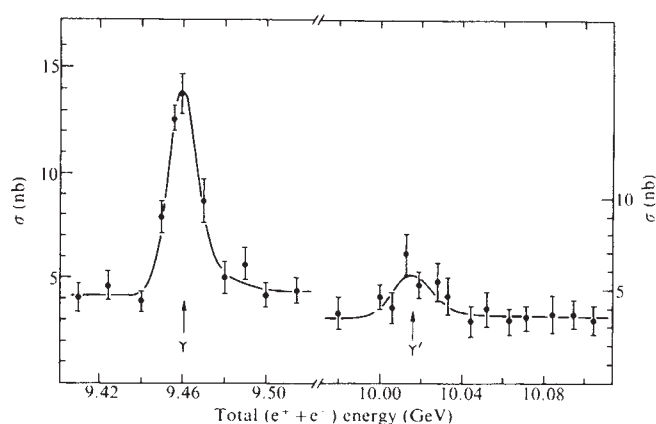


Fig. 2 Production of Y and Y' in e^+e^- annihilation at DORIS¹⁹.

Giving the quarks spin $1/2$ permits a description of hadron states embracing $SU(3)$ multiplets of different spin, within representations of the higher symmetry $SU(6)$. The quark model has been extended by including orbital (angular momentum) and radial excitations to account for higher mass and spin regions of the spectrum.

The original triplet of quarks had to be extended to a quartet (and thus $SU(3) \rightarrow SU(4)$) by the discovery of a new hadronic flavour, charm (c), in 1974. This was first signalled by the observation of a heavy, surprisingly stable meson state of mass 3.1 GeV called the J/ψ found simultaneously by the groups led by Ting¹⁰ at Brookhaven and Richter¹¹ at SPEAR. The J/ψ is formed of ($\bar{c}c$) and is accompanied by a number of other 'charmonium'^{12,13} states in a spectrum typical of that expected for a two fermion system (analogous to positronium, e^+e^-). The existence of a fourth type of quark with charge $+2/3$ had been suggested earlier by Glashow (who chose the name) and his collaborators; at first this was to achieve a quark-lepton symmetry (two pairs of each) and then¹⁴ to explain why a strangeness changing neutral-current decay mode of the K^0 -meson ($K^0 \rightarrow \mu^+ + \mu^-$) was strongly suppressed.

A fifth quark, named 'bottom' (b), (or 'beauty') is required by the experiments¹⁵ of Lederman *et al.*; this almost certainly heralds the existence of a sixth quark with a new flavour, to be called 'top' (t) (or 'truth'). Lederman's experiment¹⁶ detected a new meson state, called the Y (upsilon), as a peak at 9.5 GeV in the mass spectrum of ($\mu^+\mu^-$) pairs produced by 400 GeV protons at Fermilab. The same state has since been found^{17,18} at the DORIS e^+e^- colliding beam machine, running close to its upper limit of energy, and in pp collisions at the CERN ISR. The upsilon is partnered by at least one higher level, Y' (see Fig. 2)¹⁹ and probably a second, making a heavy analogue of the charmonium spectrum; this time the states are of hidden bottom ($b\bar{b}$) rather than hidden charm ($c\bar{c}$). The evidence points to charge $-1/3$ for this massive, ~ 5 GeV, quark and the new higher energy e^+e^- machine, PETRA, just starting to operate at DESY will be able to make states containing a single b -quark (such as, ($b\bar{d}$), and ($u\bar{b}$)).

The pattern of quark flavours, pairs of charge $-1/3$ and $+2/3$, demonstrated by the first four, is related to a quark-lepton symmetry embedded in the new unified theory of the weak and electromagnetic interactions. This is the reason for anticipating the sixth flavour, top, to partner bottom. It is not possible to predict the mass of hadrons carrying the t -quark but 'toponium' and 'exposed top' hadrons probably lie within reach of PETRA and another new e^+e^- machine called PEP, due to start up at SLAC in a year or so, both with maximum energies in the 40 GeV region.

Another important property of quark states, called 'colour', has emerged from the study of hadron spectroscopy. Certain baryons (such as the Δ^{++} state of mass 1238 MeV and spin $3/2$) contain three identical quarks with parallel spins in a state which is symmetric (the wave function does not change sign) to the

interchange of a pair of quarks. This breaks a cardinal rule, the spin-statistics theorem of Pauli, which requires the overall wave-function describing a system of identical fermions to be anti-symmetric on interchange of a pair. The difficulty has been resolved by introducing another degree of freedom²⁰, a means to distinguish the otherwise identical quarks. This requires a set of three labels say, red, blue and green. Then the forces between quarks are required to be such that at any instant each quark is a different colour (for example, the $\Delta^{++}(1238)$ state is $(u_R u_B u_G)$). The requirement that the colour part of the wave function be anti-symmetric to interchange of a pair of colour indices (to satisfy the spin-statistics rule) limits the low-lying hadron states to colour singlets—that is, states with no net colour; thus this new factor need not lead to a further great expansion of the hadron spectrum through the existence of coloured states.

Alternatively, by postulating that only colour singlet states may exist the possible quark combinations are reduced to just $(3q)$ and $(q\bar{q})$ so explaining the absence of free quarks (q), or such systems as (qq) , $(q\bar{q}q)$, and $(qqqq)$. (But quark 'molecules' such as $(q\bar{q} \cdot q\bar{q})$ or $(qqq \cdot q\bar{q})$ would be allowed and there is some tentative evidence for them).

The lifetime of the π^0 -meson provides one direct piece of evidence for colour: without it the prediction of the lifetime is wrong by a factor of 9. Colour is evidently closely related to the nature of the quark binding force, a point we shall discuss with the new theory of the strong interaction. But we now look at three different types of experiment giving evidence for the constituent quark model and further support for colour.

Lepton-nucleon scattering

Although experiments at the highest possible energies have failed to detect quarks knocked out of the nucleon, they have, nevertheless, played an essential part in creating the presently accepted picture of hadron structure. This is particularly true of the use of leptons as high energy probes of the nucleon; they are, apparently, structureless themselves and the nature of the interaction, electromagnetic for electron and muon, weak for the neutrino, is well understood.

The electron scattering experiments at SLAC late in the 1960s, and those soon afterwards in the Gargamelle bubble chamber at CERN, Geneva, using beams of neutrinos, repeated for the nucleon what Rutherford's α -particle scattering experiments did for the atom early this century (for a review see ref. 21). They found evidence that high momentum transfer collisions occurred with point-like constituents within the nucleon; moreover, the two experiments together showed that these constituents (or partons as Feynman named them) possessed just the properties expected of quarks: fractional electric charge and spin $1/2$. Furthermore, although subject to rather large errors, the neutrino experiment can 'count' the number of 'valence quarks' in the nucleon (those determining its physical attributes) and the result is close to three.

There was a significant additional observation, the quarks (plus a small component of $(q\bar{q})$ pairs forming a 'sea') only carry $\sim 50\%$ of the nucleon momentum. This forced the conclusion that the remaining 50% is carried by other constituents which display no electromagnetic or weak interaction; the obvious

speculation was that these other constituents must be associated with the strong force binding the quarks and so they were called 'gluons'. In the quark-parton model of lepton-nucleon scattering the elementary interaction with the quark can be represented by Fig. 3a and b for the electron (or muon) and neutrino cases respectively. The struck quarks do not escape as nucleon debris so it must be supposed that in some subsequent strong interaction between the quarks and gluons the quark is 'dressed' and what emerges is a jet of conventional hadrons. Nevertheless, in some average sense this jet is expected to carry information about its parent quark.

This simple point-like behaviour which implies no dependence on a fundamental length or scale and in which the quarks are treated as free, independent entities is often referred to as scaling; it leads to a linear rise with energy of the weak interaction total cross-sections. However, further experiments with electrons at SLAC, muons and neutrinos at Fermilab and recently with large detectors exposed to the high energy neutrino beams of the CERN-SPS, have revealed departures from this simple picture. This is no surprise. The linear rise must be halted before the cross-section reaches an upper bound set by reaction theory, the unitarity limit; a damping of the rise sets in when momentum transfers become comparable with the mass of the mediators of the weak interaction, but these effects are not significant in the present experiments. There is also the apparent paradox in a picture of quarks so strongly bound they cannot escape yet act quasi-free under the impulse of a high momentum transfer collision. In fact these 'scale-violating' effects, occurring at the 10–20% level, may prove to be important clues to an understanding of the interaction between quarks and gluons, now seen as the basic form of the strong interaction.

Electron-positron annihilation

The creation of lepton pairs or hadrons in the annihilation of high-energy electrons and positrons takes place through an intermediate virtual photon and there is good evidence that in hadron production the second step is creation of a $(q\bar{q})$ pair, (see Fig. 4b). There are three kinds of evidence for the quark mechanism.

First, the strength of the electromagnetic coupling to the virtual photon is proportional to electric charge and this leads to a prediction of the ratio of hadron production (via quarks) to that of muon pairs:

$$R = \frac{\text{rate to hadrons}}{\text{rate to } \mu^+ \mu^-} = 3 \sum_j e_j^2$$

where e_j is the charge of the quark of flavour j , and the 3 is due to the three quark colours. At total annihilation energies ~ 2.5 GeV, where the u , d and s quarks should contribute, the value of R is ~ 2.5 , quite close to $3[(1/3)^2 + (1/3)^2 + (2/3)^2] = 2$ which is the simple quark model prediction. Note that, for fractional charges, agreement requires the colour factor of 3, otherwise the prediction would be $2/3$. At energies ~ 5 GeV, above the threshold for production of charmed hadrons and the τ -lepton, R has risen to 4.7 ± 0.05^{22} again in reasonable agreement with the new expected value of 4.33 (1 is added by the τ -lepton and 1.33 by charm.)

A second observation^{23,24} is that the hadrons tend to emerge in two 'back-to-back' jets, presumably the progeny of the intermediate $(q\bar{q})$ pair; this effect is particularly clear at high total energies, above 5 GeV, where the angular spread in each jet is less broad. A remarkable confirmation of this comes from the angular distribution in the angle made by the axis of these two jets, with respect to the line of the e^+e^- collision; this is exactly as predicted for quarks of spin $1/2$.

Muon-pair production by hadrons

The quark-parton model is also successful in describing the creation of pairs of muons in high energy collisions between hadrons.

The mechanism proposed by Drell and Yan²⁵ is shown in Fig. 5: it is assumed that in the collision an anti-quark of the $(q\bar{q})$ sea

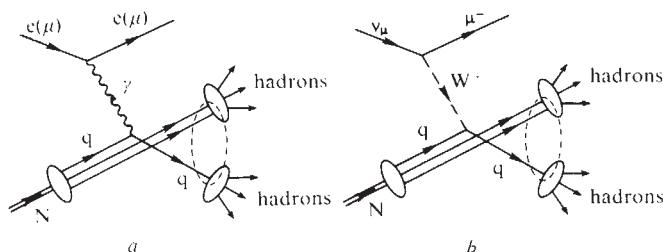


Fig. 3 a, Electromagnetic—photon exchange. b, Weak—vector boson (W^+) exchange.

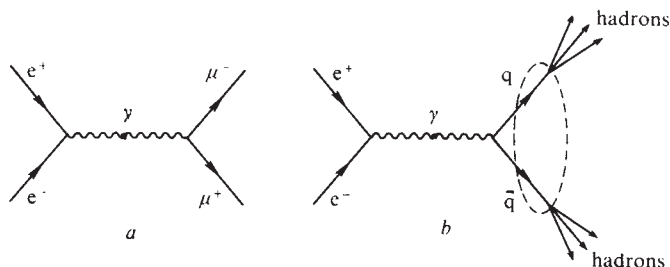


Fig. 4 a, Lepton pair production. b, Hadron production.

in one hadron annihilates with a quark in the other hadron resulting in the creation of a lepton pair. This is like a reversal of the e^+e^- annihilation to hadrons (Fig. 4b) and the probability of making muon pairs of a certain mass can be written as a product of the rate for the $(q\bar{q})$ annihilation to leptons and the probability of finding a q and $\bar{q}$ carrying given fractions of the hadron momenta. The first quantity is calculable, and already tested by the e^+e^- annihilation experiments, while the second can be related to the high energy lepton scattering experiments.

The distribution in mass of muon pairs created by 400 GeV protons at Fermilab in the experiment of Lederman^{26,27} is shown in Fig. 6. The continuum (away from the Υ region) is well described by the Drell-Yan model using q and $\bar{q}$ momentum fraction distributions for the nucleon which are quite compatible with those deduced from lepton scattering; the comparison also favours the colour factor of 3. Muon pair production provides a means of sampling the $\bar{q}$ of the sea and also the valence q and $\bar{q}$ of mesons when these are used as incident hadrons.

Thus three quite different types of experiment reflect the same internal structure and taken together with hadron spectroscopy (and a number of other phenomena not mentioned) the total body of evidence in favour of quark constituents is now overwhelming. (For recent reviews see refs 28,29.) But there are significant discrepancies, quantitative and qualitative, which show that the naïve quark-parton model is not the whole story. The next stage must be to see if these discrepancies also have a common origin, in particular, whether they are reflections of the quark-gluon interaction.

Weak and electromagnetic interactions

Like the electromagnetic force the weak interaction is believed to be mediated by spin 1 particles, called intermediate vector bosons (Table 3) which must be massive (>20 GeV) because at present energies there is no significant damping of the energy dependence of the cross section. The weak interaction responsible for β -decay, and many other processes, is well described by the V-A theory in which two forms of vector interaction (V and A) participate. This mixing of V and A causes the parity violation, or lack of invariance with respect to transformations between mirror-image situations, which is characteristic of the weak interaction.

The law of conservation of electric charge can be linked to the fact that the electromagnetic field potential is not unique or,

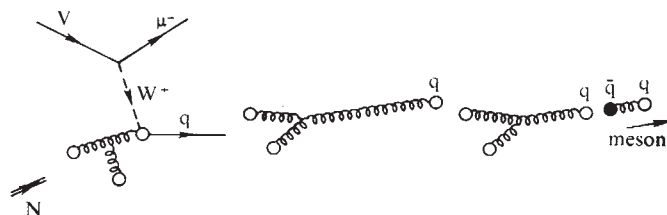


Fig. 5 Drell-Yan mechanism for $\mu^+\mu^-$ pair production in nucleon-nucleon collision.

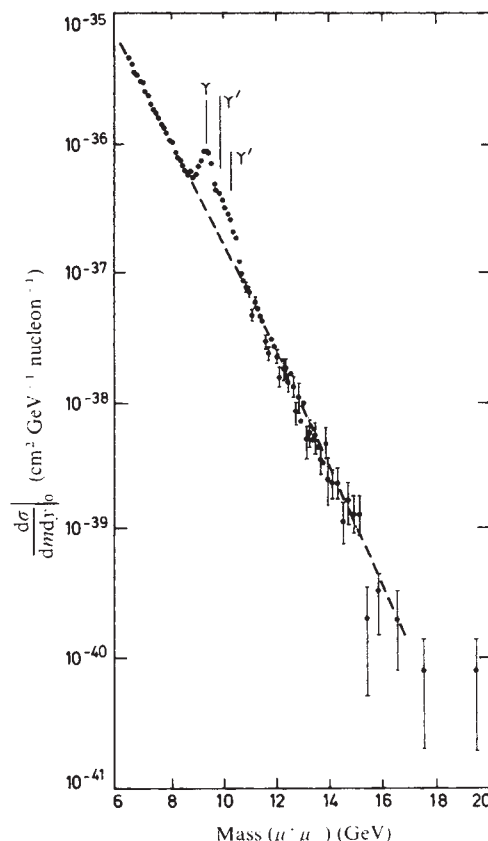


Fig. 6 The mass distribution of $\mu^+\mu^-$ pairs produced by 400 GeV protons at Fermilab^{26,27}.

equivalently, that the theory, QED, is invariant to transformations, called gauge transformations, of the unitary group $U(1)$. The electromagnetic interaction does not violate parity. Nevertheless, the vector nature of both forces made it natural to seek a theoretical framework, a gauge theory³⁰, able to describe both phenomena. However, there seemed to be at least one fundamental obstacle: the only known form of the weak interaction involved a change of charge by the participants, requiring the exchanged field particle to carry electric charge. In contrast the electromagnetic exchange current is neutral and seemed to have no counterpart in the weak interaction. Most unification schemes called for neutral as well as the familiar charged weak current.

This crucial question was settled at CERN in 1973 by the discovery of weak neutral current interactions in an experiment made by Lagarrigue and his collaborators^{31,32} using the Gargamelle bubble chamber exposed to the CERN neutrino beam. The most successful unified gauge theory is also the simplest; it is due, independently, to Salam and Weinberg³³⁻³⁵ and owes its firm theoretical foundation to the work of t'Hooft^{36,37}. Experimental support has now been assembled for this theory in various neutrino experiments and also, very recently, by an elegant experiment³⁸ carried out by Taylor *et al.* at SLAC in which minute parity violating effects due to the weak neutral current have been observed in the scattering of polarised electrons by deuterium. This provides the first test of the Salam-Weinberg theory outside neutrino induced processes and the result is in excellent agreement with those from the neutrino experiments. Attempts have been made to detect parity violation due to weak neutral current effects in certain atomic transitions and after some early null results parity violating effects have now been observed; however, the experiments are difficult and the situation remains unclear.

The Salam-Weinberg theory is a gauge theory in which the intermediate vector bosons couple to left-handed doublets (Table 4) of the quarks or leptons (or to right-handed doublets of anti-quarks or anti-leptons). (Left-handed and right-handed

describe the correlation between spin and momentum directions. A right-handed particle moves in the direction which also corresponds to a clockwise twist—like a right-handed corkscrew. The asymmetry in couplings is clearly parity-violating.) The charged current interaction, mediated by a W^+ , transforms one member of a doublet into the other, like a rotation in a ‘weak isospin’ space. These operations are those of an $SU(2)$ gauge transformation group; thus the basic gauge symmetry structure of the theory is $SU(2)_w \times U(1)_e$.

Actually the charge $-1/3$ members of the weak $SU(2)$ doublets are not identical with the flavour states d and s of hadron spectroscopy. The weak interaction couples to a mixture of s and d ;

$$d' = d \cos \theta_c + s \sin \theta_c \qquad s' = -d \sin \theta_c + s \cos \theta_c$$

where θ_c is the mixing angle, introduced by Cabibbo³⁹. (Any mixing with b is small.) This structure explains the depressed rates of decay of strange particles ($s \rightarrow u$ is proportional to $\sin^2 \theta_c \sim 0.06$) and also provides the mechanism involving the charm quark proposed by Glashow, Illiopolous and Maiani⁴⁰ to explain the extremely low rate of $K^0 \rightarrow \mu^+ \mu^-$ decay.

Table 3 The mediators					
Force	Name	Mediators	Mass (GeV)	Spin	State of theory
Gravity	Graviton		0	2	Quantised theory not established.
Weak +	Intermediate Vector Bosons	W^+	(~78)	1	(V-A)
		Z^0	(~89)	1	—
Electro-magnetic	Photon	γ	0	1	QED Salam-Weinberg almost established.
Strong	Gluon	g	0	1	QCD Attractive new gauge theory; some support but not established.

A condition which must be satisfied to ensure that unmanageable infinities do not prevent calculations with the theory takes the form, in the Salam-Weinberg case, of the requirement that the sum over the charges of the quark and lepton doublets must be zero. Table 4 shows this condition is satisfied provided there are three colours and the numbers of lepton and quark pairs are equal.

The success of the Salam-Weinberg theory is especially remarkable as it contains only one free parameter, the Weinberg angle, θ_w , which measures the relative strengths of the electromagnetic (e) and weak (g) interactions:

$$\sin^2 \theta_w = e^2 / g^2$$

$\sin^2 \theta_w$ has been determined in various experiments, including the one on electron-deuterium scattering at SLAC, and all the values obtained are close to the average, 0.23 ± 0.02 . One prediction of the theory gives the masses of the weak vector bosons; these are:

$$M(W \pm) = \frac{37.3}{\sin \theta_w} \text{ GeV} \sim 80 \text{ GeV}$$

and

$$M(Z^0) = \frac{M(W)}{\cos \theta_w} \sim 90 \text{ GeV}$$

No existing particle accelerator or colliding beam machine can reach the energies needed to create these states which thus present a major challenge for the next generation of super high energy devices. The first such opportunity will come from the proton-anti-proton collider⁴⁰ now under construction at

CERN. The aim is to store both protons and anti-protons in the CERN SPS; the two beams will circulate in opposite directions at an energy of 270 GeV so that when they are made to collide in the centre of the detecting apparatus 540 GeV will be available for the creation of W s and Z^0 s.

Table 4 Weak $SU(2)$ doublets				
Charge	Quarks		Leptons	Charge
+2/3	$\begin{pmatrix} u \\ d' \end{pmatrix}_L$	$\begin{pmatrix} c \\ s' \end{pmatrix}_L$	$\begin{pmatrix} t? \\ b \end{pmatrix}_L$	$\begin{pmatrix} \nu_e \\ e^- \end{pmatrix}_L, \begin{pmatrix} \nu_\mu \\ \mu^- \end{pmatrix}_L, \begin{pmatrix} \nu_\tau \\ \tau^- \end{pmatrix}_L$
-1/3				0
				-1

A theory for the strong interaction

A gauge theory of the strong interaction in its most elementary form, the binding of quarks in their hadrons, has been developed based on the idea that colour is the ‘strong charge’ and the force between quarks is mediated by the exchange of coloured, zero mass gluons. The gluons form a colour octet, (8), of spin 1 and the $SU(3)_c$ symmetry of colour is supposed to be exact. This theory, known as quantum chromodynamics or QCD^{41,42}, generates a force with two remarkable properties which provide a plausible basis for understanding the paradoxical behaviour of quarks and the theory is now receiving more quantitative support from experiment.

In contrast to QED, where the exchange particle carries no charge and so photons do not interact with each other (at least in lowest order), in QCD the gluons carry colour-charge and can interact strongly. This leads to a profound difference in the forces: whereas the Coulomb potential between electric charges falls at large distances ($\propto 1/r$), in QCD the force between quarks may increase; conversely the force becomes weaker at small distances, such as are probed in high momentum transfer collisions. The latter property, known as asymptotic freedom, provides a qualitative basis for understanding the success of the simple quark-parton model. On the other hand, the increase at large quark separations may be sufficient to prevent free quarks escaping from their confinement within the hadrons; instead the increase in field energy may be released in the form of created ($q\bar{q}$) pairs, or mesons. This can be visualised as shown in Fig. 7. The high momentum W imparts an impulse to the ‘almost free’ quark q ; as q moves away the binding force increases, eventually the stored energy is more than sufficient to create a new ($q\bar{q}$) pair. In effect, the ‘spring’ breaks, a new hadron is created, the quarks remain confined. Like the quarks the coloured gluons are also permanently imprisoned.

The large distance behaviour, where the quark-gluon coupling is strong, is not, so far, amenable to calculation except in a strongly model-dependent way. However, considerable success has been achieved in constructing models including quark confinement and describing the spectroscopy of hadron states^{21,30}. On the other hand, a perturbation method of calculation can be applied in the small distance, high energy regime where the coupling strength is weaker. This permits, for example, the calculation of ‘strong radiative corrections’ to the quark-parton model. As already described, the naïve version assumes the quarks are free but the first order effects of gluon coupling lead to departures from exact scaling which are calculable and so can be compared with those found by experiment.

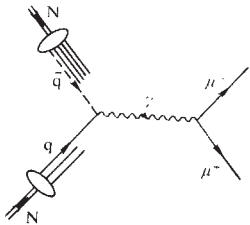


Fig. 7 Illustration of quark confinement mechanism.

These corrections are shown by examples of first order QCD processes in Fig. 8. Recent analyses of experimental data on scaling violations show good agreement on the whole with the QCD predictions. One example is the case of neutrino scattering studied by Perkins and collaborators using the BEBC and Gargamelle bubble chambers in the CERN neutrino beams. Scaling violation is demonstrated^{44,45} in this experiment by the dependence of moments of the observed quark momentum-fraction distribution in the nucleon on the momentum transfer in the collision. The results obtained are shown in Fig. 9 as plots of three different pairs of moments; the lines in this figure have slopes predicted by QCD which are in good agreement with the data. This result demonstrates that the form of the scaling violation is consistent with that predicted by QCD but more particularly the magnitude of the slopes is evidence for the vector nature of the gluon.

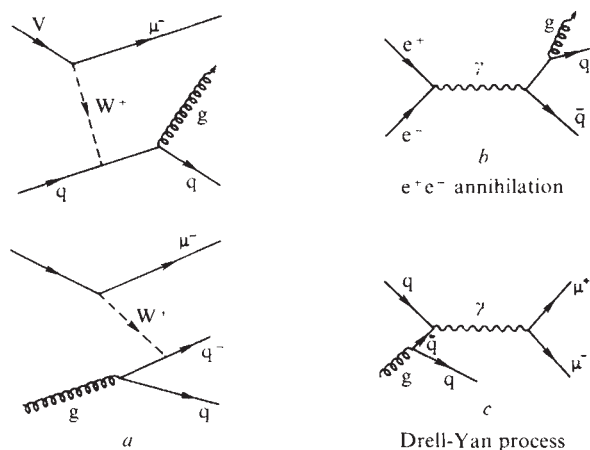


Fig. 8 Examples of 1st order QCD processes.

The first order QCD correction (see Fig. 8b) may also account for the measured values of R being rather larger than the naïve prediction. More direct evidence for gluons is being sought among the showers of hadrons created in high energy collisions, some of which should be born in gluon jets. The heavy-onium states, like charmonium or bottomonium, formed by e^+e^- annihilation are constrained to decay to hadrons through three gluons ($Y \rightarrow 3g \rightarrow \text{hadrons}$) so providing another 'laboratory' for gluon studies. So far a clear difference has been established between hadron production through Y formation and direct e^+e^- annihilation, but a proof of the intermediate role of three gluons may have to wait for the higher mass toponium.

Many other experiments are now bringing QCD under scrutiny and the present indications are that we may have, for the first time, a serious contender for a theory of the strong interaction.

Questions for tomorrow

On the experimental front further results from the polarised electron scattering experiment at SLAC may soon close (or open) one of the few remaining loop-holes left for an alternative to the Salam-Weinberg unified theory of the electro-weak interaction, a synthesis of significance equal to Maxwell's unification of the electric and magnetic forces. But the crucial tests must await the observation of the intermediate vector bosons, W^+ and Z^0 . These may be identified at the high energy colliders under construction like the CERN $\bar{p}p$ facility due to operate in 1981 using the SPS ring or the 400 GeV proton-proton collider, ISABELLE, at Brookhaven which is planned to be ready in 1986. Another contender will be the $\bar{p}p$ collider planned at Fermilab using the superconducting magnet ring being installed to double the present accelerator's energy to 1,000 GeV; this may eventually allow 2,000 GeV to be reached in the collision. However, a full study of their properties and decisive tests of the unified gauge theory through study of the

process $e^+e^- \rightarrow W^+W^-$ will need a super high energy e^+e^- collider such as the large electron positron (LEP) machine⁴⁶ now under discussion in Europe which could have an energy of up to 100 GeV in each beam.

The almost conclusive experimental support for the Salam-Weinberg theory brings another question to the fore. It is based on a gauge theory, due to Yang and Mills⁴⁷, which only applies to massless particles. The introduction of mass depends on a device called the Higgs mechanism and a consequence is the existence of at least one neutral, spin 0, particle called a Higgs meson. Its general properties are known but the mass is not predicted, it must be greater than ~ 12 GeV but may be as heavy as 1,000 GeV. The search for the Higgs meson, or at least an indirect hint of its existence, must be one of the prime objectives for the future and especially for a LEP.

The programme seems well defined, but suppose that there are no W^+ or Z^0 to be found? At the high energies of a LEP the strength of the weak interaction will overtake that of the electromagnetic force and a new view of their character is bound to be revealed.

In the field of strong interactions QCD has brought new hope of reaching and understanding although daunting problems remain, especially in the 'large distance, low energy and strong coupling' regime where the problem of quark confinement remains to be understood. Great efforts are being applied to making tests in the 'short distance' regime probed in high energy processes and for which calculations using perturbation methods can be attempted. QCD has great theoretical attraction, being a gauge theory with vector mediators it carries the promise of eventual unification with the electro-weak theory, but decisive experimental tests are going to be very difficult. The principal actors, quarks and gluons, move on a stage hidden from direct observation.

Remembering the sequence atom, nucleus, nucleon, the frontier has now moved inside the nucleon; but the old hadron spectroscopy seems to have been replaced by a new spectroscopy of quarks and leptons. How many flavours of quarks and leptons are there and is there an even deeper layer of structure to be unravelled? Only experiments can show; still more flavours may be discovered at PETRA, PEP and LEP. However, one significant sign may be the growing connection between the elementary particle states (quarks and leptons) and the character of the forces. The Salam-Weinberg theory links the quarks and the leptons; QCD loses its property of asymptotic freedom if there are more than 16 quark flavours; the

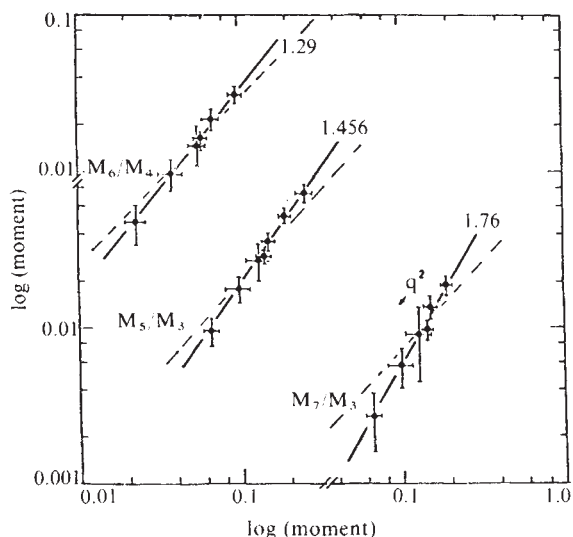


Fig. 9 Data from neutrino scattering experiment at CERN^{44,45}. The scale violating dependence on momentum transfer squared (q^2) spreads the points; the lines shown have the slopes predicted for spin 1 gluons (full-line) or spin 0 gluons (dashed line).

increase from 4 to 6 quarks is just what is wanted⁴⁸ to provide a mechanism for the violation of time-reversal invariance observed in rare decays of the K^0 -meson.

A fundamental challenge is the search for a theoretical structure which would unify the three forces within one strong-electro-weak theory. This requires a larger gauge symmetry including $SU(3)_c \times SU(2)_w \times U(1)_E$. Such a grand unification may provide answers to many outstanding questions including the relative masses of the quarks and leptons, the values of $\sin^2 \theta_w$ and the Cabibbo angle, θ_c . In these schemes the proton usually loses its absolute stability, decaying with a lifetime which may be as short as 10^{34} yr; the present limit is $>10^{30}$ yr. Beyond this lies the still greater task, left by Einstein, of achieving a unification

with gravity, the force which moulds the Universe but which is too weak for its effects to be studied at the particle level.

Most of the quarks and leptons seem superfluous; the enigma of the muon has only deepened as its new companions have arrived. To construct our world, outside the high energy physics laboratories, Nature needs only one quark pair (u,d) and one pair of leptons (ν_e, e^-). The other flavours of quarks and leptons seem to play no essential role (except perhaps the extra neutrinos which (if massless) may have helped to determine the course of the first few moments⁵⁰ following the Big Bang); they are clues to the overall scheme of Nature, which, in Salam's view, may be more sparing of principles than of particles.

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articles

Radiocarbon measurements on submerged forest floating chronologies

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The submerged forests along the west coast of England and Wales provide a unique source of wood for radiocarbon/dendrochronological studies. We report ^{14}C age determinations on sequential growth increments from three 'floating' chronologies. A sampling frequency of ~ 10 samples per century was used. Fluctuations in atmospheric ^{14}C levels of 2–3% over several decades can occur, these variations being superimposed on a smoothly changing trend.

THERE is increasing interest and controversy surrounding the issue of calibration of the radiocarbon time scale^{1–5}. The new data and interpretation presented here justify a view of moderation between the opposing extremes of opinion demonstrated by Pearson *et al.*^{1,3} and Suess² respectively.

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We report the first results of a study of past radiocarbon concentrations as reflected in ancient wood. The source of our material was the 'submerged forests' exposed at low tide around the coast of the UK. These submerged forests are the consequence of drowning, by post-glacial sea-level rise, of coastal woodlands. The trees grew at about mean high water mark, usually behind a coastal barrier of some kind, and were usually killed by the rising water table or by the ingress of salt water rather than by catastrophic overwhelming on a large scale. Preliminary radiocarbon measurements (J.A.C. and M.S.B., in preparation) have shown that there is a wide age range of wood available. As rates of isostasy and eustasy are fairly well understood, it is therefore possible, by reference to established sea-level curves⁶, to collect samples for analysis systematically, thus accelerating compilation of a tree-ring chronology. At any one site, there are large numbers of trees, mainly oak with pine and, to a lesser extent, alder, yew and birch. Thus a master chronology can be constructed, this record then being overlapped with chronologies at different locations. The trees are ideal

for dendrochronological studies; individual growth rings are wide and the growth pattern is extremely sensitive and therefore well characterised. (The dendrochronology of the submerged forests is by Heyworth at Aberystwyth.)

The submerged forests also provide excellent material for radiocarbon calibration studies. Trees of different ages all grew at the same altitude, that is, contemporary sea level and should not experience such local effects as higher ambient ^{14}C concentration and *in situ* production which may limit the previously derived bristlecone pine calibration. Plentiful supplies permit collection of large samples and a high sampling frequency, that is, 10 samples per century, with the benefit of duplication of samples and collaborative analyses to maintain the essential high level of objectivity.

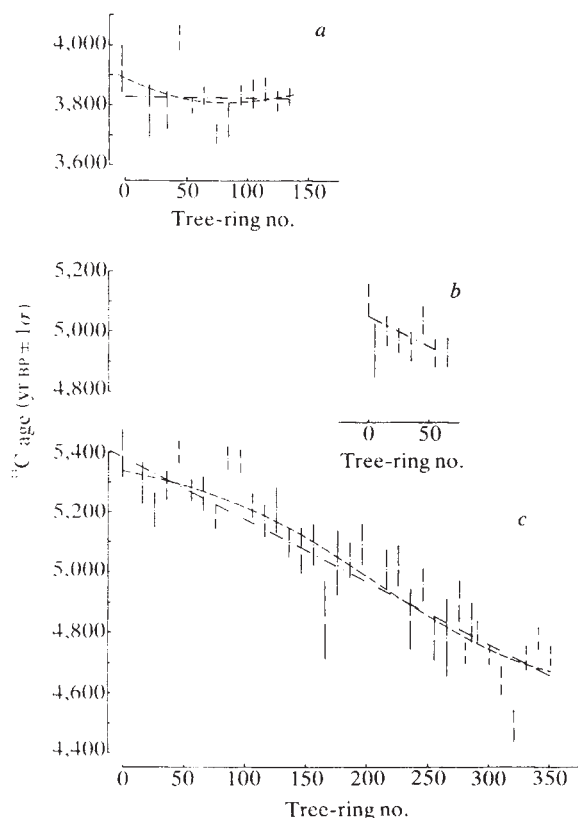


Fig. 1 Radiocarbon age (yr BP) versus tree-ring number for a, Borth 4; b, Ynyslas 1; and c, Stolford 4-5 floating chronologies. The vertical error bars indicate the 1σ counting statistics uncertainty associated with individual measurements. On the axis of abscissa age increases to the left, - - - - - Represents the linear least-squares fit, represents a least-squares polynomial approximation.

Experimental methods

Ten-year growth increments were separated by hand from the bulk sections which had previously undergone dendrochronological analysis. Individual wood samples were finely chipped in preparation for chemical pretreatment and subsequent conversion to benzene for liquid scintillation counting. Fairhall and Young⁷ have demonstrated that there can be significant differences in the ^{14}C levels of different components of wood, namely cellulose, lignin and resin. Therefore the ^{14}C assay of wood samples was performed on the cellulose fraction only, as the cellulose in each ring is not in dynamic equilibrium with the metabolism of the tree but, once synthesised, remains fixed and thereby satisfies the closed system assumption of the radiocarbon dating method.

Cellulose was extracted by digestion of the finely divided wood in aqueous 2M KOH followed by bleaching in hot HCl/NaClO₂ for 48 h. The conversion of cellulose to benzene for liquid scintillation counting was performed using standard procedures^{8,9}. Following sample combustion, an aliquot of carbon dioxide was removed for mass spectrometric analysis of

the stable carbon isotope enrichment to allow correction for natural fractionation. Subsequent radiometric measurements were normalised to account for this effect.

The possibility of artificially inducing isotope fractionation through non-quantitative chemical conversion yields was investigated (J.A.C. and M.S.B., in preparation). Aliquots of synthesised benzene were converted to CO₂ and the stable carbon isotope enrichment was measured. Only small differences in the isotopic composition between CO₂ from sample combustion and CO₂ from synthesised benzene were recorded. These differences ($\sim 0.5\%$), uncorrelated with conversion yields, do not contribute significantly to the overall precision.

^{14}C measurement was by liquid scintillation counting on a Packard series 3330 Tricarb spectrometer. Three channels monitored the ^{14}C pulse height spectrum, the positioning of discriminators minimising contributions from ^3H (to $<0.01\%$) and background pulses. The principal channel monitored the ^3H -free portion of the ^{14}C pulse-height spectrum, and the other two channels subdivided the spectrum to monitor small changes in counting efficiency induced by quenching agents. A quench correction calibration was evaluated and all count rates corrected accordingly.

Standard low potassium glass vials were used for ^{14}C counting. A sample volume of 10 ml was adopted and scintillation vials were precoated with matt black paint to this liquid level to reduce spurious cross-talk pulses. Where necessary, samples of synthesised benzene were diluted with spectroscopic grade 'dead' benzene to bring the volume up to the standard amount. The scintillation mixture contained 2 ml of a PPO/POPOP solution and vials were sealed with the supplied, high density, screw-on caps. Close monitoring of spiked samples did not reveal evaporative losses during time periods considerably longer than a typical counting cycle.

The background count rate was determined by repeated measurements on samples of benzene synthesised from materials containing no ^{14}C and samples of spectroscopic grade benzene. A rigorous study of sources of background pulses was conducted. Variability between vials induced by differences in vial weight and by small variations, ± 1 mm, in that part of the vial not masked, was insignificant. In contrast to the work of Pearson *et al.*¹ no correlation with barometric pressure was found. Over several months the variability in background count rate, for a particular vial, expressed as a standard deviation on the mean value of individual counting cycles, was comparable to the uncertainty associated with a single measurement. Hence only statistical variability in the number of counts has a role in background determinations and thus a high degree of long-term instrument stability clearly exists.

Similarly, data on individual NBS oxalic acid samples, compiled over many months, also indicated excellent long-term counter stability, the standard deviation of individual counts about the mean (2.2%) being comparable with the standard deviation of a single measurement (2.4%). Only random variability in the count rates is again found with no long-term evaporative losses. A slow increase in the degree of quenching was found but the sample channels ratio calibration corrected observed count rates. Replicate NBS oxalic acid determinations show a scatter comparable with the uncertainty on an individual count (2-3%). Thus the dominant factor in the precision of our ^{14}C age determination procedure is the counting statistics uncertainty, and errors associated with experimental procedures contribute only a small amount to the overall analytical error.

To ensure the validity of data, several intercalibration studies were carried out. These data will be published elsewhere (J.A.C., M.S.B., L.A. *et al.* in preparation) but agreement between laboratories implies satisfactory analytical accuracy.

Based on the long-term counter stability reflected in the reproducibility of background and modern standard data, and on the findings of the replicate analysis and intercalibration studies, any observed variations in the radiocarbon/tree-ring data presented here can be attributed to true changes in atmos-

pheric ^{14}C concentrations rather than to experimental variability.

Results and discussion

The results of sequential growth-increment analysis of three floating chronologies are presented in Table 1 and Fig. 1. The shortest chronology, spanning 80 yr, was pine wood from the Ynyslas exposure (YS-1, $52^\circ 29'\text{N}$, $4^\circ 4'\text{W}$), and the two longer oak chronologies from Borth, Cardigan Bay (BH-4, $52^\circ 29'\text{N}$, $4^\circ 4'\text{W}$) and Stolford, Bridgewater Bay, Somerset (SD-4-5, $51^\circ 12'\text{N}$, $3^\circ 6'\text{W}$) comprised respectively 155 yr and 360 yr. Figure 2 shows results for an additional oak timber recovered from a late prehistoric site in north-east Scotland. Analysis of these samples was shared with the radiocarbon laboratory of the Scottish Universities Research and Reactor Centre, East Kilbride. Sample preparation was performed at East Kilbride with ^{14}C counting being carried out at the Glasgow laboratory. (Details of the floating chronology are published elsewhere¹⁰.) The tree-ring numbering sequence was from the oldest growth ring to the youngest. On each radiocarbon analysis, one sigma counting errors are given based on accumulated sample counts and on established modern standard and background uncertainties. This error also includes a contribution from the imprecision of stable carbon isotope enrichment measurement required for correction for natural fractionation.

Analysis of tree-ring/radiocarbon data for possible variations in the natural ^{14}C content of the atmosphere requires subjectivity in the selection of a relationship between the two time scales and there is therefore little to choose between statistical fitting and free-hand drawing. As relatively short time periods are presented here only a simple least-squares model is considered and non-random variability about this trend line is discussed below.

Linear least-squares analysis was applied to the data. If the linear model is to be a good approximation to the results, then the mean square deviation about the regression line (MSDR)

Table 1 Radiocarbon/tree-ring data for the Borth 4, Ynyslas 1, Stolford 4-5 sequences

Laboratory code	Tree-ring no.	Radiocarbon age (yr BP $\pm 1\sigma$)	Laboratory code	Tree-ring no.	Radiocarbon age (yr BP $\pm 1\sigma$)
Borth 4 series—oak wood					
GU-742	15–10	3,917 $\pm$ 84	GU-749	81–90	3,751 $\pm$ 61
GU-743	11–30	3,781 $\pm$ 95	GU-750	91–100	3,835 $\pm$ 29
GU-744	31–40	3,783 $\pm$ 58	GU-751	101–110	3,838 $\pm$ 46
GU-745	41–50	4,027 $\pm$ 35	GU-752	111–120	3,856 $\pm$ 33
GU-746	51–60	3,798 $\pm$ 30	GU-753	121–130	3,812 $\pm$ 31
GU-747	61–70	3,832 $\pm$ 31	GU-754	131–140	3,828 $\pm$ 32
GU-748	71–80	3,705 $\pm$ 37			
Ynyslas 1 series—pine wood					
GU-711	10–10	5,106 $\pm$ 48	GU-715	31–40	4,946 $\pm$ 46
GU-712	0–10	4,935 $\pm$ 89	GU-716	41–50	5,035 $\pm$ 46
GU-713	11–20	5,009 $\pm$ 52	GU-717	51–60	4,924 $\pm$ 40
GU-714	21–30	4,974 $\pm$ 39	GU-718	61–70	4,930 $\pm$ 48
Stolford 4-5 series—oak wood					
GU-763	12–10	5,398 $\pm$ 79	GU-781	181–190	5,039 $\pm$ 61
GU-764	11–20	5,298 $\pm$ 68	GU-782	191–200	5,092 $\pm$ 67
GU-765	21–30	5,201 $\pm$ 55	GU-784	211–220	5,007 $\pm$ 61
GU-766	31–40	5,285 $\pm$ 45	GU-785	221–230	5,015 $\pm$ 68
GU-767	41–50	5,399 $\pm$ 40	GU-786	231–240	4,844 $\pm$ 97
GU-768	51–60	5,269 $\pm$ 40	GU-787	241–250	4,958 $\pm$ 54
GU-769	61–70	5,258 $\pm$ 60	GU-788	251–260	4,783 $\pm$ 67
GU-770	71–80	5,186 $\pm$ 45	GU-789	261–270	4,775 $\pm$ 124
GU-771	81–90	5,382 $\pm$ 43	GU-790	271–280	4,908 $\pm$ 68
GU-772	91–100	5,377 $\pm$ 41	GU-755	275–284	4,733 $\pm$ 34
GU-773	101–110	5,220 $\pm$ 39	GU-791	281–290	4,831 $\pm$ 67
GU-774	111–120	5,169 $\pm$ 53	GU-756	285–294	4,803 $\pm$ 36
GU-775	121–130	5,204 $\pm$ 72	GU-757	295–304	4,718 $\pm$ 31
GU-776	131–140	5,094 $\pm$ 51	GU-758	305–314	4,645 $\pm$ 41
GU-777	141–150	5,067 $\pm$ 81	GU-759	315–324	4,490 $\pm$ 47
GU-778	151–160	5,092 $\pm$ 70	GU-760	325–334	4,715 $\pm$ 31
GU-779	161–170	4,840 $\pm$ 127	GU-761	335–344	4,778 $\pm$ 42
GU-780	171–180	5,020 $\pm$ 104	GU-762	345–355	4,707 $\pm$ 32

Growth-ring numbers increase from the old to young extremes of the sequences.

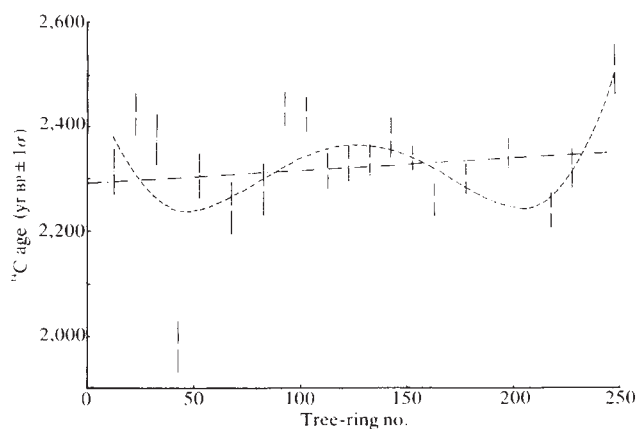


Fig. 2 Radiocarbon age (yr BP) versus tree-ring number for Cullykhan 1 floating chronology¹⁰. Vertical error bars indicate 1σ counting statistics uncertainty associated with individual measurements. --- And --- represent respectively linear and polynomial approximations to the data.

should reflect only the analytical contribution which, for the Glasgow laboratory, is dominated by the counting statistics uncertainty ($\bar{\epsilon}_c^2$). Table 2 compares the observed MSDR and $\bar{\epsilon}_c^2$ values. In the case of the short pine chronology, YS-1, the linear model is a reasonable fit to the trend line (Fig. 1), indicating that, during the time period represented by this tree-ring section, atmospheric ^{14}C concentrations were decreasing at approximately 0.5% per century parallel to growth. The linear approximation is not good in the case of oak sections BH-4, SD-4-5 and CK-1, the mean square deviation terms considerably exceeding the expected values based solely on analytical uncertainty. The trend lines for these floating sequences indicate that during the BH-4 chronology atmospheric ^{14}C concentration was decreasing at 0.8% per century whereas for the Stolford chronology the trend was one of increasing ^{14}C at 1.5% per century. Over the 250 yr Cullykhan growth period a general decrease of 1.5% per century is evident. Polynomial fitting applied to the three oak chronologies reduces the discrepancy between observed and expected values of MSDR (see Figs 1 and 2). Nevertheless, the fit remains unsatisfactory.

Visual inspection of the tree-ring chronologies indicates non-linear trends in the ^{14}C age profile. For example, the rapid increases in atmospheric ^{14}C displayed at the old and young extremes of the Stolford oak chronology are clearly non-random effects. Fluctuations such as these, amounting to 2–3% in 50 yr, have not generally been observed in previously reported bristlecone pine-radiocarbon data. This may be due to a damping effect arising from the lower sampling frequency of the American work¹¹. Reduction of the sampling frequency to 5 per century, whether as contiguous 20-yr samples or as five discrete samples, seems to smooth the Glasgow data. For example, if the Stolford data are subjected to averaging of adjacent sections to provide five samples of 20 rings per century, the MSDR decreases from 8,455 to 4,797.

Thus, for the time periods measured, the atmospheric ^{14}C concentration seems to have changed smoothly with occasional more rapid fluctuations superimposed on the gradual trends. This evidence is therefore between the traditional views either of a rapidly wriggling or almost linearly changing ^{14}C record. Periods of stability and of rapid fluctuation apparently occur in succession. While these findings apparently contradict, in particular, the results obtained from the Irish bog oak measurements, the real variability in the submerged forest data is believed to be a consequence of the increased sampling frequency used in the latter work. For the same reason, short-term variability of 2–3% over several decades is generally more rapid than those proposed in the bristlecone pine chronology.

A possible explanation for the perturbations may be that ^{14}C in wood, but not necessarily ^{14}C in the atmosphere as integrated over each year, may experience local effects. Lerman *et al.*¹² have reported small differences in the ^{14}C specific activity of tree rings from different locations. Furthermore, the work of Young

Table 2 Comparison of mean square deviation residuals

Floating sequence	Observed residual MSDR	Expected residual ($\bar{\epsilon}^2$)
Borth 4	5,577	2,016
Ynyslas 1	3,424	2,601
Stolford 4-5	8,455	3,448
Cullykhan 1	12,030	1,399

Expected value is based on counting statistics uncertainty, observed value is calculated by linear least-squares analysis of the floating sequences (Cullykhan 1 is also included).

and Fairhall¹³ on the distribution of fallout ^{14}C , has shown the existence of significant seasonal, latitudinal and meridional variations in the input of radiocarbon from the stratosphere, the source of natural ^{14}C , to the troposphere. Thus the temporal relationship between the critical periods of tree-ring growth and maximum tropopause instability is likely to be fundamentally important in the incorporation of local effects. Very rapid growth during this time of maximal stratosphere-troposphere incursion should lead to high sensitivity to short-term fluctuations in cosmic-ray-produced ^{14}C . Thus as both the tree-ring growth and stratospheric input periods are brief and tend to coincide (in spring/summer) the ^{14}C record in wood may not reflect the mean annual ^{14}C level throughout the troposphere, the latter being smoothed by atmospheric mixing. Hence, at locations at which stratospheric influx is extensive, tree rings will tend to record higher amplitude variations in ^{14}C content. Transitions from a smooth to irregular ^{14}C trend could therefore reflect either a change in solar, or, on Earth, global parameters such as geomagnetism, or could simply imply modification of meteorological conditions at the sampling site. The exceptionally slow growth of bristlecone pine wood may then give rise to a much slower response through a longer storage of nutrients. Clearly ^{14}C measurements on single tree rings from the same growth year should show some correlation with ring widths if this is a true source of local effects. Also Tans *et al.*¹⁴ have suggested that differences in ^{14}C in the cellulose fraction of tree rings from the same year but at different locations may reflect a shift in the major growth season of individual trees.

Reiter¹⁵ has shown that influxes of stratospheric air into the troposphere can be triggered by solar flares. As present-day transport of natural ^{14}C cannot be observed directly because of the effect of fallout radiocarbon, evidence must be based on the distribution of those sister cosmogenic radionuclides which were not produced in nuclear weapon tests. Thus measurements of ground-level concentrations of ^7Be and ^{32}P following such events on the Sun have shown increases of 50–100%, which are clearly not related to production rate changes. Moreover the elevated levels of these radionuclides persist for several days. If similar local and short-term enrichments of tropospheric ^{14}C occur during the tree-ring growth period, variability in the ^{14}C record in wood is quite feasible.

The full value of the floating tree-ring sequence data can only be assessed when they become fixed on an absolute scale. Statistical comparison of the submerged forest floating chronologies with a master chronology such as the bristlecone pine tree-ring record, using the method of Clark and Renfrew¹⁶, is unsatisfactory in this case because of the disparate sampling frequency. Comparisons have, however, been made for the UK data. Differences in the growth pattern of submerged forest oak and pine wood sections preclude dendrochronological overlapping of the tree-ring records. Statistical comparison of Ynyslas 1 (pine) to Stolford 4–5 (oak) shows the excellent agreement of the ^{14}C data. In Fig. 1, the tree-ring axes for these chronologies have been aligned to show this concordance. Matching of the Borth 4 (oak) chronology with a section of the Irish bog oak chronology is shown in Fig. 3. The agreement between the two floating chronologies is good. Although for the time period studied both laboratories agree about the overall trend in atmospheric ^{14}C concentrations, the higher sampling frequency of the Glasgow work suggests that there are short-term variations superimposed on this trend.

The manipulation of floating and master chronologies to produce a statistical fit requires linear least-squares analysis of the separate chronologies as well as combined least-squares analysis yielding the placement of the floating series on the master time-scale. With the Irish tree-ring sequence taken as master chronology, analysis of variance indicated that the average uncertainty associated with a Belfast radiocarbon assay, shown by the mean square deviation about the regression, is ~ 35 yr. Such a value is greater than the 25-yr analytical error reported by Pearson *et al.*¹. This ± 25 -yr uncertainty was calculated by combining error contributions assessed by laboratory standardisation (Baillie, personal communication). However, the magnitude of the uncertainty indicated by the regression analysis suggests either that the laboratory standardisation has failed to evaluate all of the uncertainty associated with the ^{14}C assay procedures, or that there is an additional, non-experimental contribution to the total variability.

The statistical fit produced by the treatment of Clark and Renfrew is limited by the precision of the radiocarbon data comprising the floating and master chronologies. Thus, the comparison of Borth 4 with the Irish chronology is limited mainly by the higher average variability of the submerged forest data. The Belfast tree-ring/radiocarbon data have also been aligned against an absolute chronology, presumably the bristlecone pine dendrochronology. This analysis, using an interactive data analysis technique (Baillie, personal communication), places the Belfast data to within 10 yr on the absolute time scale. Bearing in mind the ± 25 yr uncertainty for the floating chronology radiocarbon data and $\sim \pm 40$ yr for the bristlecone pine ^{14}C analyses, the apparent precision of the fit seems optimistic. Pearson *et al.*¹ predict an ultimate precision for their ^{14}C analyses of ± 12 yr resulting solely from an increase in counting time. At present, quoted counting errors are $< \pm 17$ yr, implying an uncertainty arising from non-counting sources of at least ± 18 yr. Hence it would not be possible to decrease the total uncertainty associated with ^{14}C assay by increasing the counting time.

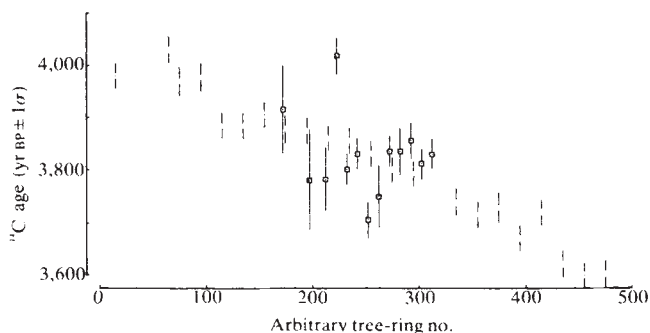


Fig. 3 Statistical comparison of Borth 4 chronology against a section of the Irish bog oak chronology. ● Represents the Irish data with associated ± 25 yr uncertainty, □ represents the submerged forest data with appropriate 1σ counting error.

In conclusion, ^{14}C measurements on floating chronologies from the UK submerged forests indicate that short-term variations in natural radiocarbon concentrations can occur. Fluctuations of 2–3% over several decades superimposed on smoothly changing trend have been detected with a sampling frequency of ~ 10 samples per century. Periods of relative stability of ^{14}C also seem to occur. Until the past ^{14}C record is precisely defined, therefore, calibration of the radiocarbon timescale must continue to involve a significant uncertainty and the accuracy of existing radiocarbon dating techniques will remain correspondingly limited.

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Crystal structure and molecular interactions of tropomyosin

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The three-dimensional structure of tropomyosin filaments has been determined by X-ray crystallography. The ends of the molecules were located by reference to the single pair of cysteine residues. Departures from the α -helical-coiled coil conformation may occur in localised domains along the molecule as well as at the overlapping ends.

TROPOMYOSIN is a key protein in the regulation of muscle contraction. The molecule is a two-chain α -helical-coiled coil that forms filaments wound in the two grooves of the actin helix. In many muscles, a calcium-sensitive protein complex, troponin, which binds to both tropomyosin and actin, controls contraction^{1,2}. Tropomyosin is present in all muscles, and most species have two populations with slightly different sequences termed α and β . Tropomyosin from rabbit cardiac muscle contains only the α subunit^{3,4}. The complete sequence of the α -chain of rabbit tropomyosin, comprising 284 residues, has been determined^{5,6}. There is a short-range periodicity of nonpolar residues, as envisaged in Crick's model for the coiled coil⁷ so that a regular pattern of interactions stabilises the structure⁸. The two identical chains in the molecule run in the same sense⁹; and have little or no relative stagger, as the single cysteine residue at position 190 can form an intramolecular disulphide crosslink (refs 10–13 and P.C. and C.C., unpublished observations). Analyses of the tropomyosin sequence^{14–17} have led to a model where the two chains are in exact register and bond through head-to-tail linkages to form filaments. A 14-fold repeat of acidic and nonpolar residues has also been demonstrated and related to muscle regulation^{14,17}.

X-ray diffraction studies of whole muscle have shown that the location of tropomyosin on the actin helix depends on the states of troponin: at low levels of Ca^{2+} , tropomyosin prevents the interaction of nucleotide-containing myosin heads with actin; when contraction is turned 'on', tropomyosin moves deeper into the actin groove, permitting the myosin heads to bind to actin^{18–20}. This work has been extended by three-dimensional reconstruction of thin filaments from electron micrographs²¹. One troponin complex binds to each tropomyosin molecule, probably about one-third of the distance from its C terminus^{22–24}. McLachlan and Stewart¹⁷ have interpreted the 14 quasi-equivalent zones on tropomyosin as two sets of alternative binding sites for actin. When tropomyosin is bound to the actin filament it would make seven half-twists in one molecular length, so that quasi-equivalent regions of the coiled coil would

link to seven identical actin monomers in the 'off' position. When the muscle is switched 'on,' the tropomyosin molecule would make a quarter-turn, and the seven quasi-equivalent sites on the molecule would interact with a second set of seven sites on actin. These arguments, necessarily speculative, assume a uniformly α -helical tropomyosin molecule that makes highly regular symmetrical interactions.

Tropomyosin crystals²⁵ have a uniquely high water content of 95% and are made up of an open meshwork of cross-connected filaments. One projection of the crystal has been solved⁹, and shows that the molecules are bonded head-to-tail to form filaments that are bent into gentle sinusoids, implying a super-coiling of the coiled-coil structure. Further studies have shown that the filaments run parallel to the body diagonals of the unit cell with an effective length of a molecule in the filament of $410 \pm 4 \text{ \AA}$ (refs 23, 26). The 385- $\text{\AA}$ axial repeat of troponin in muscle²⁷ thus indicates that there is a regular bending or 'kinking' of the filaments as they wind in the actin grooves²⁶. Small amounts of troponin can be incorporated into the crystal lattice and produce changes in unit cell parameters and molecular bending; these motions may be related to those occurring in the thin filaments of muscle^{23,26,27}.

We have determined the low-resolution structure of tropomyosin by single crystal X-ray diffraction analysis. The Bragg reflections used in this study extend to 20 $\text{\AA}$, near the limit for this crystal form, and from these data, based on a solution derived from the three centric projections of the crystal, the three-dimensional electron density map has been calculated. We describe the winding of the tropomyosin filaments in three dimensions, the precise molecular length, locations of cross connections, molecular motions in the crystal lattice, and a preliminary report on the relationship of the amino acid sequence to the observed structure, including the location and possible conformation of the molecular ends.

Preparation and X-ray photography of tropomyosin

Tropomyosin was prepared as described by P.C. and Perry⁴ from frozen rabbit cardiac muscle. The procedure is based on the method of Bailey²⁵ with the following modifications. Muscle was homogenised with an equal volume of 50 mM KCl, 10 mM Tris-HCl, 0.5 mM EDTA, pH 7.5, and allowed to stand for 30 min. After centrifugation at 1,000g for 15 min, the supernatant was discarded and the residue washed several times more to remove as much blood as possible. Dried muscle fibre was extracted with 1 M KCl, 25 mM Tris-HCl, 1 mM dithiothreitol (DDT) and 0.5 mM EDTA, pH 8.0, and ammonium sulphate fractionation cycles were carried out in 0.2 M KCl, 5 mM Tris-

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HCl, 1 mM DTT and 0.5 mM EDTA, pH 7.9. After fractionation, tropomyosin solutions were dialysed exhaustively against 1 mM DTT, and freeze dried.

Tropomyosin crystals were grown in quartz capillary tubes as previously described⁹. The final pH of the crystallising solution was 5.8. To grow mercury-labelled crystals, the cysteines of tropomyosin were reacted with approximately stoichiometric amounts of the organomercurial 2-chloromercuri-4, 6-dinitrophenol²⁸. After dialysis to remove unreacted mercurial the protein was set up to crystallise in the usual way, with the addition of 0.1–1.0 μ M mercurial for stability. Assays for free sulphhydryl groups indicated essentially complete reaction when at least 1.0 Hg per SH group was present initially.

X-ray photographs of crystals in mother liquor were taken at 2–4°C using an air-stream cooling device²⁹. Three-dimensional data were collected by screened precession photography from crystals that had been reacted with 0.6 mercurial per SH group. The unit cell parameters for these crystals were: $a = 120.0 \pm 0.5$ Å; $b = 241.7 \pm 1$ Å; $c = 296.0 \pm 1$ Å. Over 95% of the reflections with Bragg spacings greater than 20 Å were examined. Although the maximum Bragg angle for our data is only about 3°, precession angles up to 21° were required to reduce the radius of the blind region in upper-level photographs to an acceptable value. (Within one film pack R values were typically 0.08.) Least-squares scaling of all film packs produced a data set of 352 reflections with an overall R of 0.077, where

$$R = \frac{\sum_i |I_{ave} - I_i|}{\sum_i I_i}$$

Space group determination

The space group of Bailey tropomyosin crystals has been difficult to determine because of the lack of strong diffraction along the reciprocal lattice axes. The *pmg* symmetry of the [100] was unambiguously determined from X-ray patterns in conjunction with electron micrographs of negatively stained crystals (Fig. 1). Moreover, early X-ray photographs showed $h = \text{odd}$ reflections along $h00$ to be absent, so that the symmetry element along a was taken to be a twofold screw axis. The space group was therefore originally reported to be $P2_12_12$ (ref. 9). Because of the nature of the transform, however, the data along $h00$ are generally very weak. A few photographs obtained later showed observable 100 reflections, indicating that the space group was $P22_12$ (refs 23, 26). Of the many crystals recently examined, only one had observable $h = \text{odd}$ reflections.

There are two strong arguments in favour of the space group being $P2_12_12$, despite the occasionally observed weak 100. The first uses the minimum wavelength principle. The large unit cell

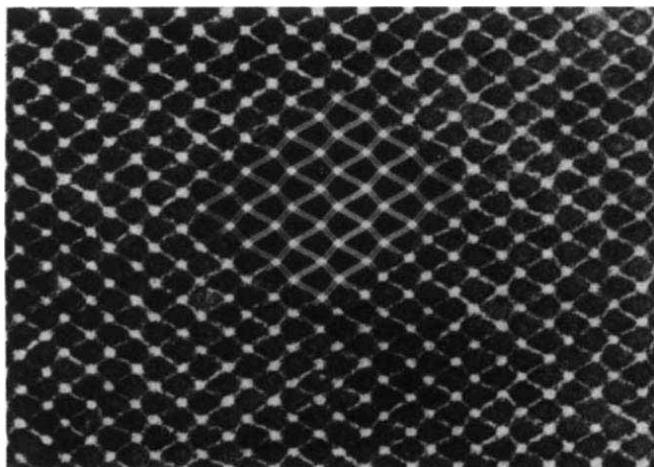


Fig. 1 Electron micrograph of a tropomyosin crystal corresponding to the view in the [100] direction. The strands form a kite-shaped net with long and short 'arms' defined by cross connections. The insert shows the pairs of tropomyosin filaments making up the strands. Negatively stained with 1% uranyl acetate ($\times 190,000$).

and localisation of the protein within 5% of the cell volume produce a reciprocal lattice sampling that is fine relative to the molecular transform, especially at very low resolution. This means that along reciprocal lattice lines perpendicular to the filament paths there can be no abrupt intensity changes except those caused by space group extinctions. The 210 and 201 reflections are always very strong whereas the 100 reflection is always either absent or weak (Fig. 2). The difference in intensity between the 100 and its neighbouring reflections is too great to be caused by changes in the molecular transform, and there must therefore be an extinction arising from the symmetry of the structure. Second, in a case where a weak 100 reflection was observed, inspection by polarised light revealed within the crystal a small crystal of a different orientation. We would attribute atypical photographs to anomalies in crystal growth. In all subsequent discussions, therefore, the space group is taken to be $P2_12_12$.

In the case of tropomyosin, the existence of screw axes instead of diads parallel to the a axis has implications about the contacts that can be made between filaments. There can be no contact in regions near the screw axes, because the filaments, which have a maximum diameter of 25 Å, are separated by one-half the unit cell, or 60 Å. This means that the bonding regions are confined to a relatively limited area of the molecule (see below).

Solution of the structure

The approximate path of the molecule in three dimensions can be deduced from the intensity distributions in the three centric projections. In special cases it is possible to solve a structure from these projections; the continuity of the tropomyosin filaments and the large solvent content make this one such a case. The projections, in fact, contain some redundancy: two projections are sufficient to determine x , y and z ; the third provides an independent check.

A net of strands has been visualised in the electron micrograph and established as the view down the a axis of the crystal (Fig. 1). The X-ray pattern could then be interpreted as arising from a pair of gently curved filaments running along the cell diagonals⁹. The cross connections between filaments produce a kite-shaped mesh for the net, so that the filaments have a long and a short arm. Note that all three views show similar intensity distributions, despite the change in scaling due to differences in lattice spacings (Fig. 2). As in the [100] projection, these patterns are indicative of sinusoids with small separations of filaments in projection. Hence, the problem of determining the three-dimensional filament path becomes one of correlating the origins of the projections. Because of the fine reciprocal lattice sampling, preliminary electron density maps can be calculated for each of the projections in a straightforward manner. For the [010] and [001] projections only three phases are required to calculate maps incorporating the ~ 12 strong reflections in each projection. The phases along the reciprocal space direction perpendicular to the filament will be positive (relative to some origin) and the phases of reflections on rows next to the diagonal will be positive on one side of the diagonal and negative on the other, the two possible choices being related by origin shifts. One has only to choose origins for the projections that produce a three-dimensional filament path consistent with the space group and the continuity of the filament. Only one solution (and its enantiomorph) satisfies these criteria. This approximate filament path was used to model the structure as discussed below.

Refinement

Tropomyosin, with its high solvent content, offers a unique method of correcting initial X-ray diffraction phases, and hence, improving the quality of the electron density map. Phases were first calculated from the Fourier transform of point scatterers placed at small enough intervals along the filament to approximate a uniform rod at 20 Å resolution. About 95% of the unit cell is solvent which must, at this resolution, have a uniform electron density, placing a powerful constraint on the phases.

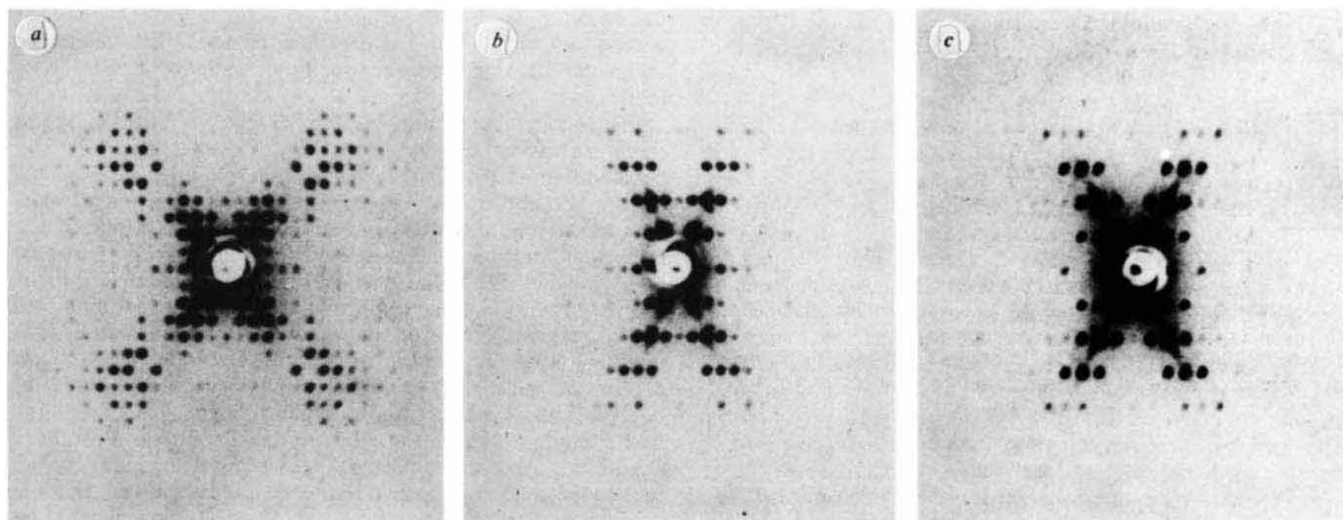


Fig. 2 Precession photographs ($\mu = 3\frac{1}{2}^\circ$) of the three principal projections of tropomyosin taken on a 10-cm camera with a double mirror focusing monochromator. Except for the lattice spacings, note the similarities of all three views. Each projection shows strong spikes arising from closely spaced pairs of filaments. *a*, [100]; *b*, [010]; *c*, [001]. In the [100] projection, nodes arising from the separation of the filaments and their curvature can be seen.

This information was included by seven cycles of setting the solvent region to a constant and calculating a Fourier transform to obtain new phases for an improved electron density map (G.N.P., in preparation). (The final *R* factor between the observed and calculated structure factors was 0.16 compared with 0.35 for the initial model.) Several views of the refined electron density map are given. Figure 3 shows the three-dimensional arrangement of filaments and their cross-connecting regions. Figure 4 shows a single filament sectioned parallel to its axis. It is relatively easy to trace the filament path through the unit cell including the area of cross connections.

Molecular length and three-dimensional winding

The tropomyosin filament winds along a line parallel to the body diagonal of the unit cell. The effective length of the molecule, that is, the repeat along the filament, can be determined to the limit of accuracy of the unit cell parameters. This length was previously estimated from projections as 410 ± 4 Å. As the path can now be traced in three dimensions, we can establish this length more precisely as 410.3 ± 1.4 Å. This information can be used to deduce the number of residues involved in the end-to-end bonding region. Assuming an average axial translation of 1.49 Å per amino acid residue in the α -helix of a perfect coiled coil, the molecular overlap is predicted to be $284 - 410.3/1.49 = 8.6 \pm 1.0$ residues. The three-dimensional winding of the filament is not, in fact, helical, but has the form of an ellipse when projected down the axis of the molecule. The sense of this supercoiling cannot yet be established. The tropomyosin filament is wound in the grooves of the actin helix, with a right-handed sense; and, for illustrative purposes we have chosen right-handed supercoiling of the filaments in the crystal.

Location of the Cys 190 residue

Rabbit cardiac tropomyosin consists of two identical chains of known sequence; there is one cysteine per chain^{5,6}. These SH groups have now been located within the unit cell. X-ray diffraction data to 20 Å have been collected from both Hg-labelled and reduced forms of tropomyosin for two projections, corresponding to views down the [100] and [001] axes. Difference Fourier maps were calculated using the phases obtained by the model building and refinement procedures. A single site is observed in each projection (Fig. 5). (Previous problems with difference Fourier maps of Hg derivatives have been accounted for by the presence of small amounts of

contaminating proteins within the lattice.) The redundancy of measurement of the value of γ provides a check on the solution; the values obtained for γ agree to within 6 Å. Difference Patterson maps were also calculated to test the validity of the solution. Major peaks were found corresponding to heavy atom positions, although other strong peaks were also present. Efforts are underway to determine the usefulness of including isomorphous replacement data in the structure analysis. The fact that there is a single peak for the two SH groups of the molecule supports a structure where the two chains are in register, although the resolution of the difference Fourier maps does not rule out a small stagger (~ 10 Å) (see below).

The Hg serves as a marker for the location of Cys 190 along the filament, but leaves a twofold ambiguity, however, in the direction of the polypeptide chain in the filament. If the N to C direction is chosen, as shown in Fig. 4, the intermolecular overlap would occur in the short arm of the filament near a crossover region. The map shows a relatively dense peak at precisely that point. This feature presumably corresponds to the end-to-end molecular overlap. The alternative choice of direction for the polypeptide chain would place the molecular overlap

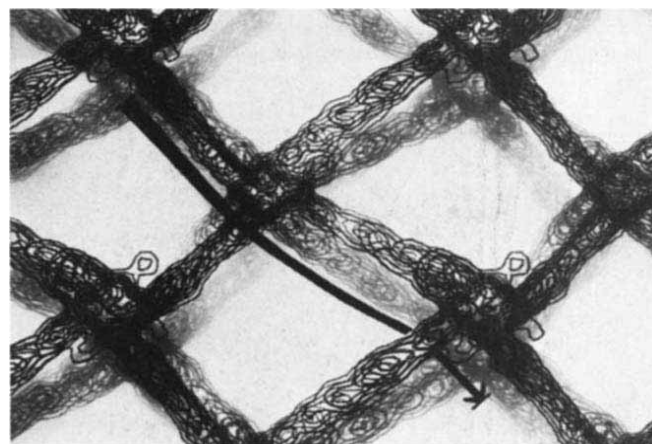


Fig. 3 The three-dimensional electron density map of tropomyosin. Several unit cells are shown at a slight angle to the [100] direction to illustrate the packing of filaments in the lattice and the location of a single molecule in the structure. (The head and tail of the arrow represent the C and N termini, respectively.) Contour values are at equal increments, the lowest of which is about 0.34 electrons Å⁻³.

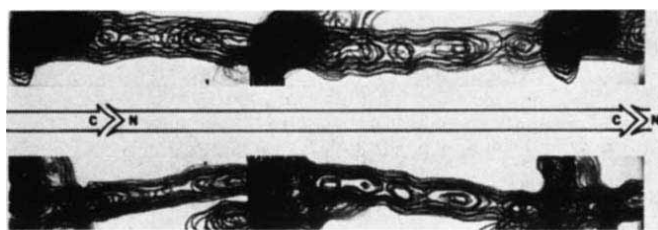


Fig. 4 A filament sectioned along its length. The top and bottom views are rotated by 90° about the filament axis. Contour values are the same as in Fig. 3 and sections are at intervals of 4 Å. Slightly more than one effective molecular repeat is displayed. The arrow shows the direction of the polypeptide chain and the location of the molecular ends.

within a crossover region and does not readily account for the electron-dense region in the short arm. Sequence information can also be used to distinguish these possibilities. A line projection of the electron density was calculated by integrating cross sections of the filament in regions where no crossovers were present. The corresponding 'theoretical' distribution was estimated based on the amino acid sequence and a 9-residue overlap. Relative weights for each amino acid were derived from Hulmes *et al.*³⁰, with the exception of cysteine, which was corrected to include its labelling with Hg. Figure 6 shows the theoretical and observed density profiles for both chain directions. The better fit of the two curves is clearly represented by Fig. 6a and confirms the solution shown in Figs 3 and 4. Introducing a stagger of seven residues did not produce a significant effect, and hence cannot be ruled out. The Hg labelling of β -tropomyosin (which contains an additional pair of SH groups) or a molecule containing an α and a β chain should allow us to confirm this solution of the chain direction.

Molecular structure

We can interpret certain additional features observed in the electron density map on the assumption that the preceding analysis gives the correct direction of the polypeptide chain. We should note initially that one might expect to establish certain parameters of the coiled coil at this resolution. The theoretical molecular cross section is, however, approximately 20×15 Å. This small difference is not discernible in the structure that we have so far achieved, and we have not, therefore, been able to determine the pitch of the coiled coil, its sense of twist or its phase in the unit cell. (As α -helices are right-handed and the coiled coil is left-handed, when we are able to visualise the twist,

the absolute hand of the supercoiling can be determined.) By comparing the limited intensity data to 15 Å with calculated molecular transforms it may soon be possible to visualise these features.

Inspection of the map shows that the run of the filament seems quite continuous (Fig. 4), including the dense region that we have identified as the molecular ends, where a head-to-tail overlap of about nine residues must take place. Here the filament shows only a slight swelling in all directions. If the molecular ends were simple α -helical-coiled coils linked by overlapping of the 'broad faces' (ref. 17), we would expect to see a discontinuity in this region of the map corresponding to a lateral stagger. However, an alternative explanation of this feature is that the molecule might not maintain the α -helical conformation at the ends so that the chains would be joined by intermeshing to form a small globular domain. This kind of link is consistent with both the electron density map and chemical characteristics of the molecule. Predictions of secondary structure based on the amino acid sequence indicate, in fact, that the ends of the chain may not be α -helical; this is particularly true for the sequence at the C terminus, whereas the N terminus does not allow a strong prediction (Argos, personal communication). Tropomyosin has been seen as the archetype of α -helical-coiled coil, but its functional design to form filaments by end-to-end bonding may have produced a specialised structure for the molecular ends.

The general smoothness of curvature of the molecular filaments—apart from the ends—implies that there are no major deviations from coiled coil structures at 4 °C. However, this observation does not mean that there are no local regions of 'flexibility'. The filament seems to be slightly kinked in several places in the map. These deviations from a uniform structure may be more pronounced at physiological temperatures.

The molecular filaments can be traced relatively easily through the cross-connecting 'knots' in the lattice. There are two regions of the molecule where intermolecular contacts are made; one is near the C terminus, and the other is about 140 Å from the N terminus (Fig. 4). (These 'sticky' regions on the molecule may also be used for cross connections in other polymorphic forms of tropomyosin²³.) Although we can now identify some of the residues involved in the intermolecular bonding, we wish to establish the direction of the molecule in the filament unambiguously (by the use of β -tropomyosin) before reporting the detailed relationship between the amino acid sequence and the electron density map.

Molecular dynamics

The tropomyosin crystal is unique not only because of its high water content but because it is made up of filaments rather than discrete molecules. Moreover, there is only limited contact between filaments in the lattice. Correspondingly, these crystals display some unusual structural features. There are considerable variations in the *b* and *c* parameters of the unit cell that have been shown to be related to the presence of other proteins in the lattice²³. We have confirmed and extended these findings. Despite these unit cell differences, however, the length of the body diagonal remains 402 Å. This figure is the projected molecular repeat along the tropomyosin filament, and its constancy implies that the molecular structure and precise end-to-end overlap are maintained despite small variations in molecular bending that occur in unit cells of different sizes.

A striking aspect of the diffraction patterns is the intense diffuse scattering seen in unscreened photographs. This is due to various types of disorder in the crystal (see ref. 31). The most plausible explanation is that this feature arises from vibrations of the filaments in the crystal lattice. In three dimensions, the diffuse scatter consists of 'disks' normal to the direction of the long arms; there is also a weaker streak associated with the short arm of the filament. This finding implies that the conformation of the long arm of the filament is relatively variable, but that there is also some vibration in the short arm (Fig. 4). This

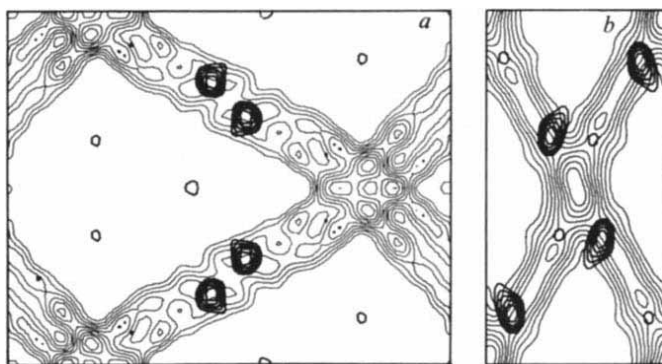


Fig. 5 Difference Fourier projections (dark lines) superimposed on the electron density map (light lines) showing the location of the Hg and, hence, Cys 190. *a*, [100] projection; *b*, [001] projection. The difference maps are contoured at values corresponding to the range 2σ – 4σ , where σ is the r.m.s. deviation in the map.

interpretation is consistent with the following experiments: X-ray photographs have been taken of crystals at 30 °C rather than 4 °C. In these conditions, reflections arising from the long arms but not those from the short arms are essentially eliminated. Moreover, this effect is reversible. This result confirms the difference in stability of the two arms of the filament and shows that the magnitude of the effect is considerable at physiological temperatures.

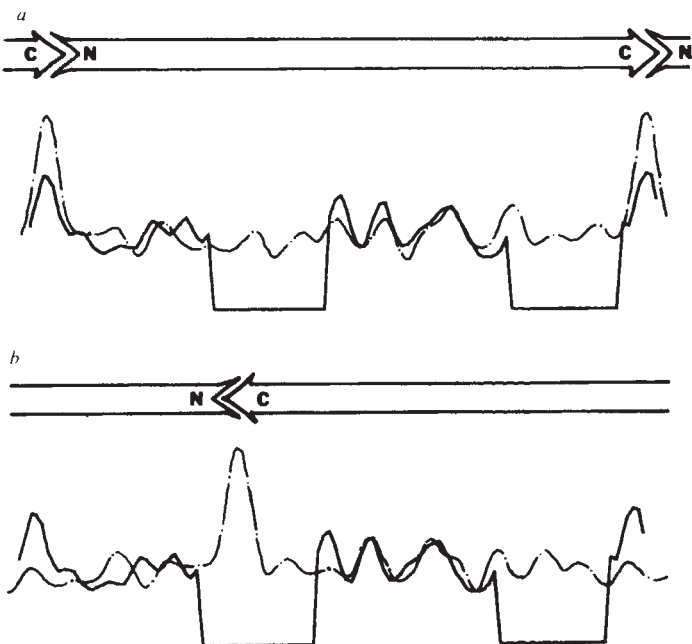


Fig. 6 Comparison of the observed (solid lines) and calculated (broken lines) electron density profiles of the tropomyosin filament for the two possible directions of the polypeptide chain. The observed electron density is taken as zero where cross-connecting filaments prevent integration of the molecular cross section. The calculated profiles were obtained by assigning weights to each amino acid in the sequence and convoluting with a gaussian of width appropriate to match the resolution of the observed curve. Curves in *a* correspond more closely than curves in *b* and the chain direction has, therefore, been taken as in *a*.

Relevance to muscle

Our results provide insight into the role of tropomyosin in muscle regulation. The molecular length we have established allows an estimate of the filament's radius of bending when it is bound on the actin helix. Taking the projected axial repeat of tropomyosin as 385 Å, this average radius can be calculated to be 45.2 Å ($(410.3^2 - 385^2)^{1/2} / \pi$). Assuming the same sense of twist, the radius of supercoiling of the molecular filament in the crystal lattice is about one quarter as large as that of tropomyosin on the actin helix. Interactions with actin, therefore, play an important part in determining the precise conformation(s) of tropomyosin in muscle. There must be some local flexibility of the tropomyosin structure to accommodate this large difference in radius of bending. The slight irregularities of the filament seen in the electron density map and the motions of the filament deduced from the diffuse scattering analysis imply localised flexible regions of the molecule³²⁻³⁴ and also indicate that there may be regions of the molecule other than the ends that are not in stable α -helical conformations.

The motions of tropomyosin during regulation depend on the precise structure(s) of the molecule and its specific interactions with actin. Attempts to determine the design of tropomyosin

have led to the recognition of some rather subtle structural features. The sequence results are consistent with an almost fully α -helical-coiled coil molecule; however, certain small regions may depart from this conformation, either in a conserved fashion (at the molecular ends) or only transiently, depending on varying interactions. The effective length of tropomyosin spans seven identical monomers on the thin filament, but there is no sevenfold repeat in the sequence; rather, 14 quasi-equivalent regions have been detected, which differ, however, rather markedly. (A single region of the molecule, for example, binds troponin.)

The presence of the high proportion of α -helical-coiled coil, and the quasi-equivalent bonding regions have led to models for tropomyosin that tend to stress its regular symmetrical features. This is, perhaps, a deceptively simple view. The kind of picture that is now beginning to emerge from our crystallographic analysis, and other studies, is that of a somewhat less regular and rigid structure. In its regulatory role in muscle, the tropomyosin supercoil moves on the actin helix. The molecular motions displayed by tropomyosin supercoils in the crystal lattice may reflect to some extent the real nature of the dynamic elements in the structure (see refs 23, 26). It is likely that at some stage of the contractile cycle, the tropomyosin molecule may be rather loosely wound about the actin helix, perhaps held strongly at only a few sites, and locally kinked at other regions. In view of the unusual characteristics of the molecule that have been deduced thus far, we suggest that the motions of tropomyosin in muscle depend on two aspects of its design: the regular coiled coil structure and quasi-equivalent regions that interact with actin; and the aperiodic features. These uncommon domains may play a critical part in the function of this highly specialised molecule.

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Endogenous mammary tumour virus DNA varies among wild mice and segregates during inbreeding

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Proviruses of the mouse mammary tumour virus (MMTV) endogenous to normal mice can be identified by molecular hybridisation and distinguished using restriction endonucleases. Feral mice display marked heterogeneity with respect to the number of copies and the sites of insertion of endogenous MMTV-specific DNA, with occasional mice apparently free of MMTV DNA. Several different MMTV proviruses present in laboratory mice have segregated like stable, independent genetic elements during the inbreeding which followed a cross between Bagg albino and DBA mice 60 years ago. The results favour the hypothesis that endogenous proviruses have been established by multiple, independent infections of germ cells rather than by somatic mutation of ancestral proviruses or of cellular genes.

DNA related to the genomes of retroviruses and capable of directing synthesis of viral gene products has been found endogenous to many animal species^{1,2}, but the origin and function of endogenous proviruses remain relatively obscure. Since retroviruses are extremely widespread in nature and have a very similar genetic organisation in different hosts, they are likely to have a common origin. It has generally been argued that endogenous proviruses have evolved either by alteration of cellular genes⁴ or by rare infection of the germ line(s) of early vertebrates⁵. To account for the retention of functional proviral DNA during speciation, it is attractive to propose that virus-specific DNA serves a function which confers some selective advantage to the host. It is also possible, however, that contemporary retroviruses evolved as viruses from a single progenitor virus and that endogenous proviruses were occasionally established by infection of the germ lines of individuals from diverse species. This proposal does not imply that endogenous proviruses have any utility for the host; their wide distribution might be simply a consequence of the ability of retroviruses to infect germinal cells, thereby introducing stably inherited proviral DNA.

Several features of the mouse mammary tumour virus (MMTV) make it suitable for investigating some of these issues. Pathogenic strains of the virus are transmitted in laboratory mice either as infectious agents in the milk of certain animals or as endogenous proviruses integrated into germ line DNA (refs 6, 7). Molecular hybridisation tests indicate that laboratory and feral mice may harbour approximately 3–15 copies of MMTV DNA per diploid genome^{9–12}; MMTV DNA has also been detected in Asian mice⁹, but little if any viral DNA has been found in rodents outside the genus *Mus*¹³.

Endogenous MMTV DNA differs markedly among feral mice

To characterise virus-specific DNA endogenous to normal cells, most investigators have relied on assays which measure the rate

of formation and stability of hybrids between labelled viral nucleic acids and cellular DNA, often using cellular DNA pooled from several individuals. Restriction endonucleases which recognise sequences within and adjacent to virus-specific DNA allow important refinements in the analysis of viral DNA: fragments generated from internal regions can be used to differentiate closely-related proviruses, and fragments which include adjacent host DNA can be used to identify the location of the proviruses in host DNA¹⁴. Since the recognition sites for these enzymes are short sequences (4–6 base pairs in length), they are likely to be conserved among members of a single species; the single copy DNA of individual mice, for example, appears to manifest less than 1% overall difference in base sequence¹⁵. We have used restriction endonucleases to compare endogenous viral DNA among individual animals, as a means of determining the origin of the DNA. If, for example, MMTV DNA had evolved from element(s) present in a progenitor of *Mus musculus* at a rate similar to that of other non-reiterated DNA, individual mice would be expected to appear similar when analysed for restriction fragments bearing virus-specific sequences.

To perform such tests, we prepared DNA from the livers of 12 wild mice trapped in four geographically distinct areas in California. (The liver is relatively resistant to horizontal infection by MMTV. Using tests for new proviruses in tissues of susceptible animals receiving virus in milk, no new viral DNA could be measured in livers in conditions adequate to detect infection of 1% of the cells¹⁶. Hence we have assumed here that proviruses in hepatic DNA represent only genetically transmitted DNA.) Following digestion with the restriction endonucleases *Pst*I and *Eco*RI, the DNA samples were assayed for fragments containing virus-specific sequences using agarose gel electrophoresis and the DNA transfer method¹⁷. These two enzymes were chosen because mapping studies with both viral DNA introduced into cells by acute infection¹⁸ and virus-specific DNA endogenous to inbred mice (see below; also ref. 16 and our unpublished results) indicated that *Pst*I recognises several sites within proviral DNA, whereas *Eco*RI cleaves at a single site (Fig. 1). Hence *Pst*I would be expected to generate several virus-specific fragments whose size would depend solely on sites within viral DNA, while cleavage of each provirus with *Eco*RI would normally produce two fragments whose size depends on sites in flanking cellular DNA. This approach has been used to show that MMTV proviruses introduced by experimental infection are located at many sites in cellular DNA (as manifested by variations in the size of *Eco*RI fragments), but that the same small region of viral DNA is always joined to cellular DNA^{16,19} (see legend to Fig. 1).

Analysis of the *Pst*I digests of wild mouse DNA (Fig. 2) showed all of the tested mice to be unique with respect to their MMTV proviruses, although fragments of certain sizes (for example, molecular weights of 3.3, 3.1, 2.5, 1.1, 0.9, 0.6, and 0.5×10^6) could be found in several of the digests. It is presumably significant that these fragments are observed in digests of DNA from inbred strains (Fig. 1; also Fig. 4, Table 1); we infer that units of viral DNA with very similar or identical internal sequences are variably distributed throughout the feral (and inbred) mouse population. We noted with particular interest the

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widespread occurrence (Fig. 2, lanes D, E, F, G, J, L, and M) of the constellation of *Pst*I fragments of 2.5, 1.1, 0.9 and 0.6×10^6 Mr also associated with the provirus acquired by infection with MMTV commonly isolated from milk (Fig. 1); in at least one case (lane F), these fragments appear to be present in the absence of any others.

The most striking finding, however, was the virtual absence of MMTV-related DNA in two of the 12 specimens examined with *Pst*I (Fig. 2, lanes H and I); annealing with a labelled MMTV-specific DNA revealed only a faint band, corresponding to a fragment of 9.0×10^6 MW common to all DNAs. Annealing of DNA immobilised on the same filter with labelled cDNA specific for rRNA demonstrated fragments common to all digests, including those in lanes H and I (bottom panel, Fig. 2), thereby excluding the possibility that the poverty of MMTV-specific bands in lanes H and I was due to some mechanical impediment to annealing. Moreover, several additional wild mice from the same location (Lake Casitas, California) have been found free of MMTV DNA (unpublished results of J.C.C. and M. Gardner). The common 9.0×10^6 MW fragment in annealings with both MMTV- and rRNA-specific cDNAs indicates a minor contamination of our viral cDNA with ribosomal sequences.

Examination of MMTV proviral DNA in feral mice with *Eco*RI produced grossly heterogeneous patterns (Fig. 3). Again, all tested mice were different; but in the *Eco*RI digests few, if any, fragments were common to two or more individuals, and the fragment sizes were not identical to those encountered in digests of DNA from inbred mice (see below, Fig. 5). One sample found to be free of MMTV-specific *Pst*I fragments (Fig. 2, lane I) also proved to lack *Eco*RI fragments which react with MMTV-specific cDNA (Fig. 3, lane I). The other negative

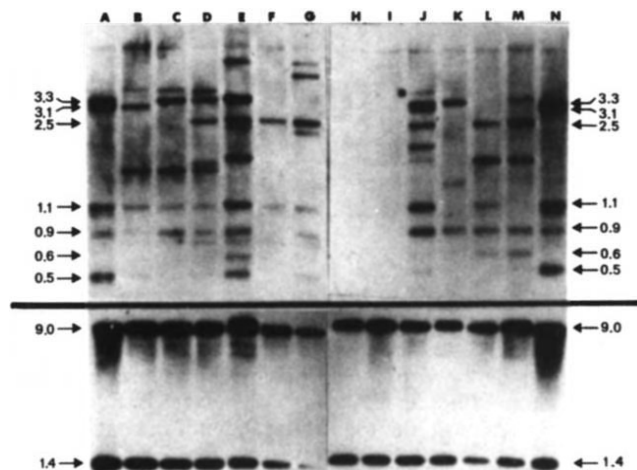


Fig. 2 Products of *Pst*I digestion of MMTV DNA endogenous to feral mice. DNA was extracted from the livers of individual wild mice using established procedures^{9,16}. Samples of 10 µg were digested to completion with *Pst*I (prepared as described by Greene *et al.*⁴¹) and subjected to electrophoresis (1–2 V cm⁻¹) in horizontal 3–6 mm slab gels of 0.8% agarose (Seakem) in buffer containing 0.04 M Tris-acetate, pH 8.15, 0.02 M Na acetate, 0.018 M NaCl, 0.002 M Na₂EDTA. After staining with 5 µg ml⁻¹ ethidium bromide and subsequent inspection under UV illumination, the DNA was transferred to sheets of nitrocellulose (Schleicher and Schull) using the procedure of Southern¹⁷ as previously described^{16,18}. Filters were baked *in vacuo* for 2 h at 80 °C, incubated at 41 °C in a pre-annealing mixture containing 50% formamide, Denhardt's solution⁴², 20 µg ml⁻¹ alkali sheared and denatured salmon sperm DNA, 0.05 M HEPES buffer, pH 7.0, and 3 × SSC (SSC = 0.15 M NaCl, 0.015 M Na citrate), then incubated 60–72 h at 41 °C in a minimal volume of a similar mixture initially containing 1–2 × 10⁶ c.p.m. ml⁻¹ of MMTV ³²P-cDNA (upper panel). Following the washing and autographic procedures (see below), the filter subsequently was incubated with 1–2 × 10⁶ c.p.m. ml⁻¹ ribosomal ³²P-cDNA (lower panel) first at 68 °C for 1 h to denature hybrids with MMTV-specific cDNA and then at 41 °C for 60–72 h. These cDNAs were prepared as described by Shank *et al.*¹⁸ using oligomers of calf thymus DNA to prime synthesis randomly on either MMTV 20–35S RNA or 18S and 28S rRNA²⁷. After incubation with specific cDNAs the filters were rinsed briefly at 23 °C with 2 × SSC, incubated at 50 °C for 1–2 h with two changes of 0.1 × SSC, 0.1% SDS, and washed frequently at 23 °C with 0.1 × SSC. Kodak Royal X-omat film was exposed to the filter using Cronex 'Lightning Plus' intensifying screens (Dupont) for up to 10 d at –70 °C⁴³. *Hind*III fragments of ³²P-DNA were included as MW standards (not shown). The MW × 10⁻⁶ of major viral-specific fragments are shown on the left for lanes A–G and on the right for H–M. *Pst*I fragments of MMTV DNA endogenous to BALB/c mice, lanes A and M, and from Californian wild mice⁴⁴ obtained from Bouquet Canyon, lanes B, C, and D; San Francisco, lanes E, F, and G; Lake Casitas, lanes H, I, and J, and Norwalk, lanes K, L, and M.

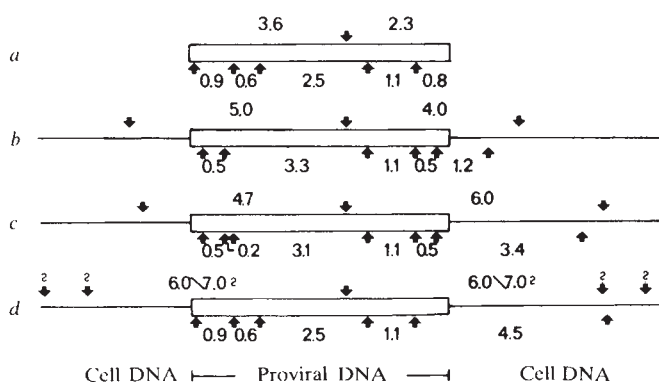


Fig. 1 Restriction endonuclease maps of MMTV DNA. The sites of cleavage of MMTV DNA and adjacent cellular DNA by the two enzymes used in this report are indicated by arrows (pointing down for *Eco*RI sites, up for *Pst*I sites). The numbers denote the molecular weights × 10⁻⁶ of consequent fragments. *a*, Linear form of unintegrated MMTV DNA in cultured cells infected by milk-borne virus from mouse strains GR or C₃H (refs 16 and 18). The linear form is approximately colinear with a subunit of viral RNA, with the right-hand half of viral DNA representing the 3' half of viral RNA¹⁸. Integrated MMTV DNA in cells infected by these viruses is colinear with the unintegrated linear species and has been found inserted in many different positions in host genomes^{16,19}. (We have not attempted to illustrate the many possible restriction endonuclease sites in cellular DNA adjacent to viral DNA introduced by experimental infection.) *b*, *c*, and *d*, Units of MMTV DNA found endogenous to certain inbred mice of the Bagg albino × DBA lineage (Fig 6, Tables 1 and 2). The maps for Unit II (*b*) and Unit III (*c*) were determined by secondary cleavages of isolated *Eco*RI fragments of BALB/c mouse DNA; details will be presented elsewhere (unpublished data of J.C.C., J. Majors, and H.E.V.). The *Pst*I fragments of 1.2 (Unit II), and 0.2 and 3.4×10^6 Mr (Unit III) could be visualised well only after annealing with cDNA specific for the 3' terminus of viral RNA and were not therefore used routinely to identify these proviruses. (cDNAs specific for either end of viral RNA anneal to both ends of viral DNA^{18,19}, indicating that terminally redundant sequences are present in MMTV DNA in a manner similar to that described for avian sarcoma virus DNA^{37–40}.) The *Pst*I map of Unit IV of endogenous MMTV DNA (*d*) is identical to the *Pst*I map of proviruses acquired by infection with milk-borne viruses (*a*). The question marks indicate uncertainty about the assignment of *Eco*RI sites in flanking cellular DNA to the right or left side of the provirus.

sample was not tested with *Eco*RI. Although the MMTV proviruses in feral mice have internal similarities, as judged by the shared, relatively small *Pst*I fragments (Fig. 2), they are presumably located at different positions in cellular DNA, as judged by the variability of the size of the *Eco*RI fragments (Fig. 3). (The large sizes of these fragments supports our prediction that at least one of the determining sites is in flanking host DNA.) The results are most readily interpreted as evidence for variable numbers of independent integrations of MMTV proviruses at various sites in host DNA. The situation appears analogous to that in MMTV-infected tissues and cells, with viral DNA inserted at different sites in host DNA in each independently infected cell^{16,19}.

We have considered the possibility that some of our results could reflect a high degree of mutability of DNA surrounding endogenous proviral DNA, with relative conservation of the internal regions (as reflected in results of digestion with *Pst*I (Fig. 2) and by previous thermal denaturation studies⁹). However, this interpretation cannot account for the absence of proviral

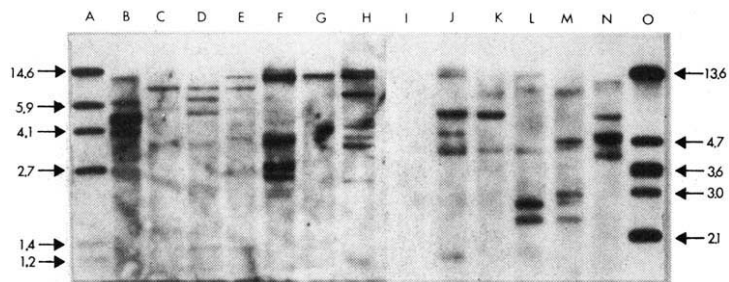


Fig. 3 Products of *EcoRI* digestion of MMTV DNA endogenous to feral mice. DNAs from the feral mice analysed in the experiment shown in Fig. 2 were digested with *EcoRI* (a gift from P. Greene) and analysed as described in the legend to Fig. 2. *HindIII* fragments (lane A) and *EcoRI* fragments (lane O) of phage λ 32 P-DNA served as MW standards for lanes B–H and I–N, respectively. *EcoRI* fragments of MMTV proviral DNA from BALB/c mice, lanes B and N; Bouquet Canyon, lanes C, D, and E (lanes B, C, and D, respectively in Fig. 1); San Francisco, lanes F, G, and H (lanes E, F, and G, respectively in Fig. 1); Lake Casitas, lanes I and J (lanes I and J, respectively in Fig. 1); and Norwalk, lanes K, L, and M (lanes K, L, and M, respectively in Fig. 2).

DNA in some wild mice or for what seem to be major differences in the number of units of viral DNA among the wild mice (for example, compare the numbers of virus-specific *EcoRI* fragments in lanes F, H, J, and M with those in lanes C, E, G, and K in Fig. 3). In addition, the required frequency of change in the hexanucleotide recognition sequences for *EcoRI* in *Mus musculus* DNA would greatly exceed the base sequence divergence expected among individuals within a single species of *Mus*^{15,20}.

Units of endogenous MMTV DNA segregate like stable genetic elements during inbreeding

Our results could also be seen as evidence of a marked propensity of retroviral DNA to undergo genetic gymnastics, including deletion, translocation, rearrangement, and amplification, in manner similar to certain elements in bacteria²¹ or predicted for retroviral DNA in the provirus hypothesis⁴. (The latter hypothesis cannot, by itself, account for the absence of viral DNA from wild mice, but we consider the possibility that it might be operative in conjunction with other mechanisms for radical genetic change.) We have been able to perform a provisional test of this explanation by examining whether multiple units of endogenous MMTV DNA have segregated like stable genetic elements, retaining their characteristic restriction endonuclease recognition sites, during the almost 60 yr of inbreeding which followed the mating of a Bagg albino female with a DBA male in 1920 (ref. 22).

Figures 4 and 5 illustrate the patterns of virus-specific fragments in *PstI* and *EcoRI* digests of DNA obtained from the livers of mice from several contemporary colonies descended from the progeny of the initial Bagg albino $\times$ DNA mating. Despite several obvious differences among these digests (to be considered below), the similarities are very striking, especially when they are viewed in comparison with the digests of DNA from feral mice (Figs 2 and 3). As the tested mice were obtained from branches of the genealogical tree that had been separated for nearly 60 yr (see Fig. 6, ref. 22), the apparent stability of restriction sites suggested that neither viral DNA nor flanking cellular DNA was highly mutable and that the elements of endogenous DNA might be amenable to segregation analysis.

To simplify this analysis, we correlated the presence of *EcoRI* and *PstI* fragments in digests of each sample, as summarised in Table 1. In making these correlations we took advantage of mapping data from related studies. (1) We knew from studies of cells and tissues infected by milk-borne MMTV¹⁶ that *PstI* fragments of 2.5 and 0.6×10^6 MW could be used to distinguish provirus acquired by horizontal infection from proviruses found endogenous to certain inbred mice (for example BALB/c). As shown here, several other strains derived from the Bagg albino $\times$ DBA mating (C₃H/Bi, CBA/J, and CBA/St; Fig. 4) carry a provirus indistinguishable with this enzyme from the provirus acquired by infection with milk-borne MMTV (Fig. 1a, d). This provirus (Unit IV in Table 1) is also associated in these mice with the appearance of *EcoRI* fragments of 7.0 and 6.0×10^6 MW; Unit IV can be found in contemporary DBA/2 mice and presumably was carried by the DBA male participating in the original cross. (2) We have generated physical maps of the MMTV DNA endogenous to contemporary BALB/c (Fig. 1b, c; Table 1) by performing secondary digestions with several endonucleases of isolated *EcoRI* fragments of BALB/c liver DNA bearing viral sequences (data not shown). These studies validated several of the associations we were able to make on genetic grounds between *PstI* and *EcoRI* fragments, and they revealed the structure of three units of endogenous viral DNA (I–III) which differ from the provirus (Unit IV) resembling DNA acquired by infection with milk-borne MMTV. These three units include two (Units II and III) very similar in complexity and organisation to the proviruses acquired by infection with milk-borne virus (that is 5×10^6 – 6×10^6 Mr); the third (Unit I) bears viral sequences with about one-half the complexity of a unit length provirus (Table 1).

These approaches led to the definition of six units of endogenous MMTV DNA which seem to be segregating in the progeny of Bagg albino $\times$ DBA; the restriction fragments associated with each unit are listed in Table 1 to facilitate inspection of the digests shown in Figs 4 and 5. When each of the contemporary colonies was scored for carriage of each of the six units, we were able to define six genotypes (A–F, Table 2). No strain carried less than two units of endogenous DNA, but only one unit (Unit II) was present in the DNA of all the laboratory mice

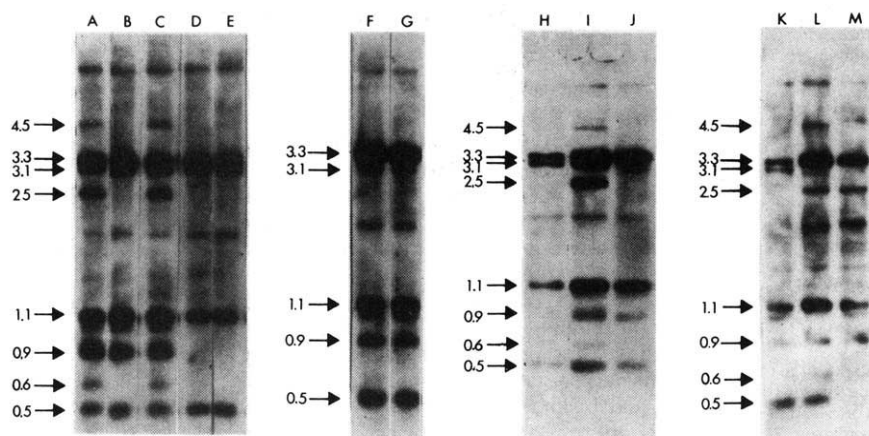


Fig. 4 Products of *PstI* digestion of MMTV DNA endogenous to progeny of Bagg albino $\times$ DBA. 10 μ g of DNA from the livers of individual mice from the strains indicated were analysed as described in Fig. 2. *HindIII* fragments of λ DNA were used as MW markers and the MW $\times 10^{-6}$ of major MMTV-specific fragments are indicated to the left of each gel. Lane A, C₃H/Bi; B, C₃H/St; C, CBA/J; D, CBA/CaJ; E, CBA/Ht-6; F, C₃H/HeN; G, C₃H/HeJ; H, CBA/N; I, C₃Hf/Bi; J, C₃H/An; K, BALB/c; L, DBA/2; M, CBA/St.

Table 1 Restriction fragments derived from six defined units of endogenous MMTV DNA

Units of endogenous MMTV DNA	<i>Pst</i> I fragments	<i>Eco</i> RI fragments
I	0.9*	10.0
II	3.3, 1.1, 0.5	5.0, 4.0
III	3.1†, 1.1, 0.5	6.0, 4.7
IV	4.5, 2.5, 1.1, 0.9, 0.6	7.0, 6.0‡
V§	3.1†	3.9¶, 3.5, 2.7
VI§	—	5.4, 9.0, 1.0**

The *Pst*I and *Eco*RI fragments useful for the identification of each of the six units of endogenous MMTV DNA observed in the progeny of Bagg albino × DBA are listed according to molecular weight ($MW \times 10^{-6}$). For discussion of the definition of the units, see text. The restriction endonuclease maps of Units II, III, and IV are illustrated in Fig. 1.

* The 0.9 *Pst*I fragment is diagnostic of Unit I when it appears in the absence of Unit IV.

† *Pst*I fragments of 3.1 are derived from Units III and V, but the corresponding band is equal in intensity to the 3.3 *Pst*I band in the first case, but is less intense in the second (compare lanes G and H in Fig. 4).

‡ Two *Eco*RI fragments, derived from Units III and IV, migrate at 6.0×10^6 Mr as shown by analysis of CBA/St (Table 2; Fig. 5, lane L) in which Unit IV but not III is present and a 6.0×10^6 MW fragment is still observed.

§ These units have not been characterised fully by biochemical techniques.

¶ The 3.0 *Eco*RI fragment is inferred from the broadening of the 4.0 band (see lane G compared with lane I in Fig. 5).

** This small molecular weight fragment is not included on the gel shown in Fig. 5.

examined here; results with feral mice (see above) indicate that this unit is not, however, ubiquitous in mice. When the genotypes were correlated with the genealogical tree for the mouse colonies used (Fig. 6), three units (I, III, and IV) were found in both major branches of the tree but not in all the members. Unit V was identified in colonies on one branch and in contemporary DBA/2 mice. Unit VI was also found in DBA/2 mice but not in any of the progeny from the original Bagg albino × DBA cross; we cannot say whether it is likely to have been present in the DBA male used in the initial cross. These results indicate that the parental Bagg albino and DBA animals were not both homozygous for any of the defined units of endogenous MMTV DNA, with the probable exception of Unit II; Units I, III, IV, and V (and perhaps VI) have consequently segregated during inbreeding.

MMTV genotypes can be used to monitor breeding programmes

To ensure that the observed results were not the result of errors in breeding protocols, we examined several colonies of some strains maintained at widely separated locations. Colonies of C3H/He mice maintained at Jackson Laboratories (Bar Harbor), the National Institutes of Health (Bethesda); and the University of California (Berkeley) yielded identical results, as did colonies of C3H/Bi (and C3Hf/Bi) from Simonson Laboratories (Gilroy, California), the Sloan-Kettering Institute (New York), and the Imperial Cancer Research Fund (London) (data not shown).

An interesting exception to the general rule of identity among separately maintained colonies was provided by the differences in genotype between the C3H/St mice maintained at the L.C. Strong Foundation (San Diego, California) and a subline, C3H/StWi, derived in 1947 by Wilson from the original Strong colony²³ and maintained at the National Institutes of Health (Bethesda); Unit IV is present in the colony derived by Wilson but not in that kept by Strong (data not shown). Since these colonies were separated in the 1940s it is probable that the animals were not fully inbred at that stage and were heterozygous with respect to Unit IV. It is, of course, also possible that Unit IV either was lost from the Strong colony as a consequence of deletion or introduced into the Wilson subline by an inadvertent interruption in the pedigree.

In view of the result with C3H/St, we looked for evidence of incomplete inbreeding or genetic instability in the contemporary colony of C3H/St at San Diego by testing the DNA from several individuals for endogenous MMTV DNA. So far no differences

have been observed among members of this colony, in tests that have included 20 individuals. In addition, analysis of endogenous MMTV sequences among numerous BALB/c colonies has not revealed any differences. These results suggest that contemporary mouse strains are truly homozygous at most or all viral loci, as predicted from other kinds of analysis²², and that endogenous MMTV DNA seems stable by the criteria which revealed marked heterogeneity among feral mice. Such tests for defined patterns of endogenous viral DNA may be a particularly sensitive assay for the success of an inbreeding programme; for example, we would have been likely to have measured the heterogeneity in C3H/St at the time of separation of its colonies (see above).

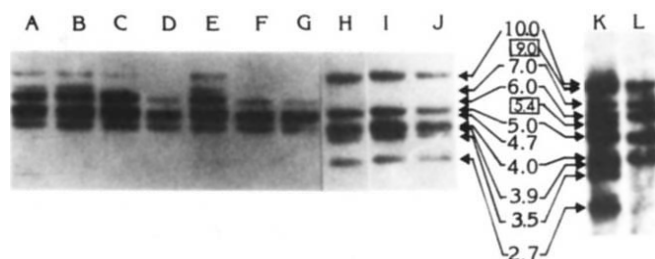


Fig. 5 Products of *Eco*RI digestion of MMTV DNA endogenous to progeny of Bagg albino × DBA. 10 µg of DNA from the livers of individual mice from the strains indicated were digested to completion with *Eco*RI and then analysed as described in Fig. 2 legend. ³²P-labelled *Eco*RI fragments of λ DNA included in the gel were used as MW standards in obtaining $MW \times 10^{-6}$ listed between lanes J and K for major viral fragments. Viral fragments found only in the DBA/2 (lane K) parent strain are presented in the squares while fragments shared by more than one strain are indicated by the larger numbering. Lane A, C3Hf/Bi; B, C3H/Bi; C, C3H/St; D, CBA/N; E, CBA/J; F, CBA/CaJ; G, CBA/Ht-6; H, C3H/HeJ; I, C3H/HeN; J, C3H/An; K, DBA/2; L, CBA/St.

Implications for origins of endogenous DNA

Our results are most readily interpreted as evidence favouring the hypothesis that endogenous proviruses result from multiple, independent, but presumably infrequent infections of germ line tissues with retroviruses. According to this view, the similarities among retroviruses from many species would reflect their probable evolution from a single progenitor virus; the rationale for their ubiquity would be no more obvious than the rationale for the ubiquity of any other of the several far-flung classes of virus; and their appearance as endogenous viruses would be a consequence of their capacity to establish their genomes in infected cells, presumably including germ cells, as proviruses covalently integrated into host cell DNA. Several ancillary observations are in accord with this proposal. (1) The four MMTV proviruses endogenous to the A strain mouse are located on more than two chromosomes²⁴, consistent with their acquisition by multiple, independent infections. (2) Evidence for trans-species transmission of viruses now recovered as endogenous viruses argues that they were established by infection of germinal tissues^{5,25,26}. (3) Avian leukosis virus-specific DNA cannot be detected in two types of wild jungle fowl, despite its presence in a third type of jungle fowl, in closely related domestic chickens, and in more distantly related pheasants (D.

Table 2 Genotypes observed in progeny of Bagg albino × DBA

Genotype designation	Units of MMTV DNA						Example
	I	II	III	IV	V	VI	
A	+	+	+	+	+	+	DBA/2 (Figs 4L, 5K)
B	+	+	+	+	+	+	CBA/St (Figs 4M, 5L)
C	+	+	+	+	+	+	BALB/c (Figs 2A, 3B, 4K)
D	+	+	+	+	+	+	C3H/Bi (Figs 4A, 5B)
E	+	+	+	+	+	+	CBA/CaJ (Figs 4D, 5F)
F	+	+	+	+	+	+	C3H/HeN (Figs 4F, 5I)

The units of endogenous MMTV DNA defined in Table 1 were sought by examination of *Pst*I and *Eco*RI digests of liver DNA from mice in colonies derived from Bagg albino × DBA (see Figs 4, 5). The presence of a unit is indicated by +, its absence by -. Six genotypes, designated, A, B, C, D, E, and F, were observed in the tested samples. An example for which complete data are provided is listed for each genotype.

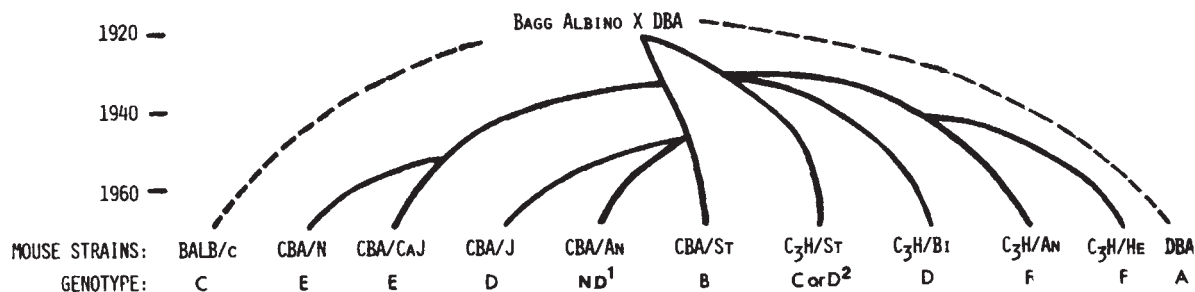


Fig. 6 Segregation of endogenous MMTV proviruses during inbreeding of progeny of Bagg albino $\times$ DBA. The genealogy of mouse strains was derived from the summary prepared by Staats²² and plotted against the recorded dates for mating and segregation. Solid lines are used for the progeny of the Bagg albino $\times$ DBA mating; dashed lines indicate the ancestors of contemporary BALB/c and DBA colonies, but are not meant to imply that the animals involved in the indicated cross were the direct predecessors of BALB/c and DBA/2 mice used here. The genotype (see Table 2) for each strain examined was determined from analyses of the sort shown in Figs 4 and 5 and is noted below the name of the strain.

* Not done because the strain was not available.

† C3H/St is assigned 'C or D' because two colonies gave different results (see text).

Frisby, R. Weiss, M. Roussel and D. Stehelin, personal communication). The lack of concordance of viral DNA with established phylogenetic relationships again argues for infection of germ lines after speciation. (4) Biochemical studies of normal chickens^{27,28} and mice^{29,30} indicate considerable heterogeneity from animal to animal^{27,28} or from strain to strain^{29,30} with respect to the amount and/or structure of endogenous leukaemia virus-related DNA. There is, however, one notable difference between results reported here and the results of studies of endogenous avian leukosis virus-specific proviruses in domestic chickens: all of the tested chickens shared one common proviral element, despite the variable distributions of other elements^{27,28}. This observation might mean that domestic chickens have been derived from a relatively small genetic pool, or it might imply that a least one provirus was introduced into the germ line of chickens at some relatively early time. (5) In the studies of avian viruses, the variability in number, location, and structure of endogenous proviruses contrasted with uniform or nearly uniform results obtained with reagents specific for cellular genes, such as globin, ovalbumin, ovomucoid, and transferrin²⁷; these findings further support the thesis that endogenous proviruses either originate or evolve in a manner fundamentally different from cellular genes.

Our results obviously run counter to the widely held view that endogenous viral DNA has evolved either from some ancient cellular sequences⁴ or from an ancient provirus present in early vertebrates⁵. This point of view has encouraged examination of the relatedness of endogenous proviruses in attempts to assess the evolutionary relationships among many animals³¹, including man³². The proposal favoured by our data, if supported by similar studies of other kinds of proviral DNA, would challenge the assumptions that underlie such studies. Certainly it is possible that some kinds of proviral DNA other than MMTV DNA appeared in germ lines, by infection or other mechanisms, sufficiently long ago to serve as useful markers for vertebrate evolution. In addition, interpretation of our data would be difficult if retroviral DNA exhibits a propensity to undergo deletion, rearrangement, or translocation. Although our results with the progeny of Bagg albino $\times$ DBA mice argue for stability of germinal proviral elements over a 60-yr period, we cannot discount rare events of these kinds over a much longer period as explanations for some or all of our observations with feral mice. Future experiments with other endogenous proviruses and with proviruses acquired by infection of cultured cells may be helpful in judging such possibilities.

Implications for mammary tumorigenesis

The hypothesis that endogenous proviruses arise by infection rather than by conservative evolution from sequences common to primitive vertebrates does not favour any functional role for viral DNA in the growth and development of normal organisms. Nevertheless, endogenous viral DNA may significantly alter

major biological processes in ways that have yet to be recognised; certainly its presence may have pathological consequences for the host animal. This idea has been dramatically documented in the laboratory by the markedly increased frequency of leukaemia in BALB/c mice following the introduction of the provirus of the Moloney strains of murine leukaemia virus as an endogenous provirus³³. In the case of MMTV, the GR strain is apparently prone to early mammary tumours⁷ as a consequence of its carriage of endogenous provirus(es) indistinguishable in tests described here from that acquired by infection with milk-borne virus of known oncogenic potential (our unpublished data). The GR mouse may be a special case, in that the provirus in question seems to be present in multiple, probably closely-linked copies^{9,10,34,35}. The biological effects of a similar endogenous provirus (Unit IV) present in some of the mice studied here remains to be determined. It is known, however, that several strains of mice develop mammary tumours late in life under the influence of genetically transmitted viruses⁶⁻⁸; examination of the DNA from such tumours by methods described here indicates that the tumour cells bear additional copies of proviruses similar to that endogenous provirus termed Unit II (our unpublished data). Presumably some alteration in the control of expression of such proviruses results in virus spread and subsequent tumorigenesis, with the pathogenic potential of the element augmented when it is integrated into a new site after secondary infection. A similar mechanism has been proposed to account for the regulation of expression of an endogenous virus of chickens³⁶. This suggests that the site at which an endogenous provirus is integrated in cellular DNA may have a considerable influence upon its expression; depending upon the consequences of viral gene expression for the host, the entry of new proviruses into the germ line at certain sites could therefore provide a selective advantage or disadvantage to the host.

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Nucleotide sequence of cloned cDNA for bovine corticotropin- β -lipotropin precursor

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The nucleotide sequence of a 1,091-base pair cloned cDNA insert encoding bovine corticotropin- β -lipotropin precursor mRNA is reported. The corresponding amino acid sequence indicates that the precursor protein consists of repetitive units and includes a third melanotropin sequence in its cryptic portion. Pairs of lysine and arginine residues separate the component peptides of the precursor.

THE pituitary hormones corticotropin (ACTH) and β -lipotropin (β -LPH) and their gene provide a useful system for studying the structure and regulation of hormonally regulated genes. These two hormones, which are formed from a large common precursor protein^{1–8}, contain small component peptides with biological activity; α -melanotropin (α -MSH) and corticotropin-like intermediate-lobe peptide (CLIP) are derived from ACTH⁹, whereas γ -lipotropin (γ -LPH), β -melanotropin (β -MSH), endorphins and methionine-enkephalin are elaborated from β -LPH^{10,11}. The intracellular level of the mRNA coding for the common precursor protein is depressed by glucocorticoids, which probably act at the transcriptional level by means of the glucocorticoid receptor^{12,13}. As ACTH and β -LPH together account for only one-third to one-half of the molecular weight of the precursor protein, there has been considerable interest in and speculation about the primary structure and possible biological functions of the remaining portion.

Recently developed techniques of DNA cloning¹⁴ and nucleotide sequence analysis^{15,16} together provide a useful approach to an understanding of the primary structure of eukaryotic genes and the regulation of their expression. Previously¹⁷, we described the construction of bacterial plasmids that contain the nucleotide sequence for the bovine ACTH- β -LPH precursor and presented the DNA sequence coding for ACTH and a portion of β -LPH. Here, we report the complete nucleotide sequence of a 1,091-base pair cloned cDNA insert that contains a duplex copy of the ACTH- β -LPH precursor mRNA. Our results define the precise locations of ACTH and β -LPH in the precursor protein and predict the amino acid

sequence of its remaining portion. They further indicate that the precursor protein consists of several repetitive units separated by pairs of basic amino acid residues, lysine and arginine, and that a third melanotropin sequence is located within the cryptic portion of the molecule.

Sequencing strategy

The bacterial plasmid pSNAC20 was constructed by inserting *in vitro*-synthesised double-stranded cDNA for the bovine ACTH- β -LPH precursor into the *Pst*I endonuclease cleavage site of the plasmid pBR322 with the use of poly(dG)·poly(dC) homopolymeric extensions¹⁷. Nucleotide sequence analysis of the inserted cDNA was carried out by the procedure of Maxam and Gilbert¹⁵; the sequence map is shown in Fig. 1. The restriction fragments used were obtained either from intact pSNAC20 or from the cDNA insert (including poly(dG)·poly(dC) tails), which was isolated by cleavage of the plasmid at the regenerated *Pst*I sites¹⁷.

For accurate prediction of the amino acid sequence of the cryptic portion of the ACTH- β -LPH precursor protein, the nucleotide sequence was determined on both strands of the cDNA for all but 33 residues corresponding to the 5' end and 47 residues corresponding to the 3' end of the mRNA; for these regions this was technically difficult, but the sequence data were reliable. In addition, care was taken to overlap the sequence data derived from different endonuclease-generated fragments. These procedures provided a useful verification of the sequence. Furthermore, some of the endonuclease-generated fragments were analysed for nucleotide sequence in duplicate or in triplicate. Thus, at least three sets of sequence data were obtained for all segments of the cloned cDNA, thus eliminating any ambiguity resulting from closely lying bands of chemically degraded DNA, which were occasionally observed on electrophoretic analysis.

Another potential problem in the use of cloned cDNA for sequence analysis of mRNA is a possible error caused by deletion, addition or rearrangement of nucleotides during *in vitro* cDNA synthesis or DNA cloning itself^{18–20}. Thus, an independent method, such as analysis of protein sequence or mRNA sequence, should preferably be used to verify that the

lation product may result in overestimation of the molecular weight of the protein by electrophoretic analysis³². The assignment of the translational initiation site at the methionine codon mentioned above seems plausible because the first 20 amino acid residues starting with this putative initiative methionine (residue -131), which are followed by the characteristic sequence Ser-Met-Glu---- (see below), include a large number of hydrophobic amino acids (13 nonpolar residues including 7 leucine residues). A high content of hydrophobic amino acids is known to be present in the signal peptides of other proteins destined for secretion³³⁻³⁶. On the other hand, if the previous estimation of the molecular weight of the precursor protein is correct, the translational initiation site would be located 1,125-1,295 nucleotides (950-1,120 nucleotides for the coding region plus the 175 noncoding nucleotides we have identified at the 3' end) away from the first residue of the poly(A) sequence of the mRNA and therefore would be absent from the cloned cDNA segment we have analysed. In Fig. 2, we have assigned amino acid residues up to the putative initiative methionine, although it remains possible that the region of the mRNA proximal to this site is translated.

Computer analysis of the amino acid sequence determined from the pSNAC20 clone indicates that the sequence of amino acid residues -55 to -44 is strikingly similar to the sequences of α -MSH and β -MSH, which are known to have common structural and biological features³⁷ (Fig. 3). This segment of the precursor protein shares with the known MSHs tyrosine and methionine residues as well as the characteristic tetrapeptide sequence His-Phe-Arg-Trp, which is required for melanotropic activity, at equivalent positions³⁸. Moreover, the peptide fragment discovered by our DNA sequence analysis is flanked on both sides by a pair of basic amino acid residues. This suggests that the new peptide, like α -MSH or β -MSH, can be formed by proteolytic processing of the precursor; by analogy with α -MSH, it is possible that phenylalanine amide is formed at the carboxyl terminus of this peptide during processing. In view of the remarkable structural homology between the newly found peptide fragment and α -MSH or β -MSH, we propose to name the peptide γ -melanotropin (γ -MSH). Another peptide fragment within the precursor protein that exhibits some structural similarity to the MSHs is that containing amino acid residues -111 to -105 (Fig. 3). Note that this fragment contains

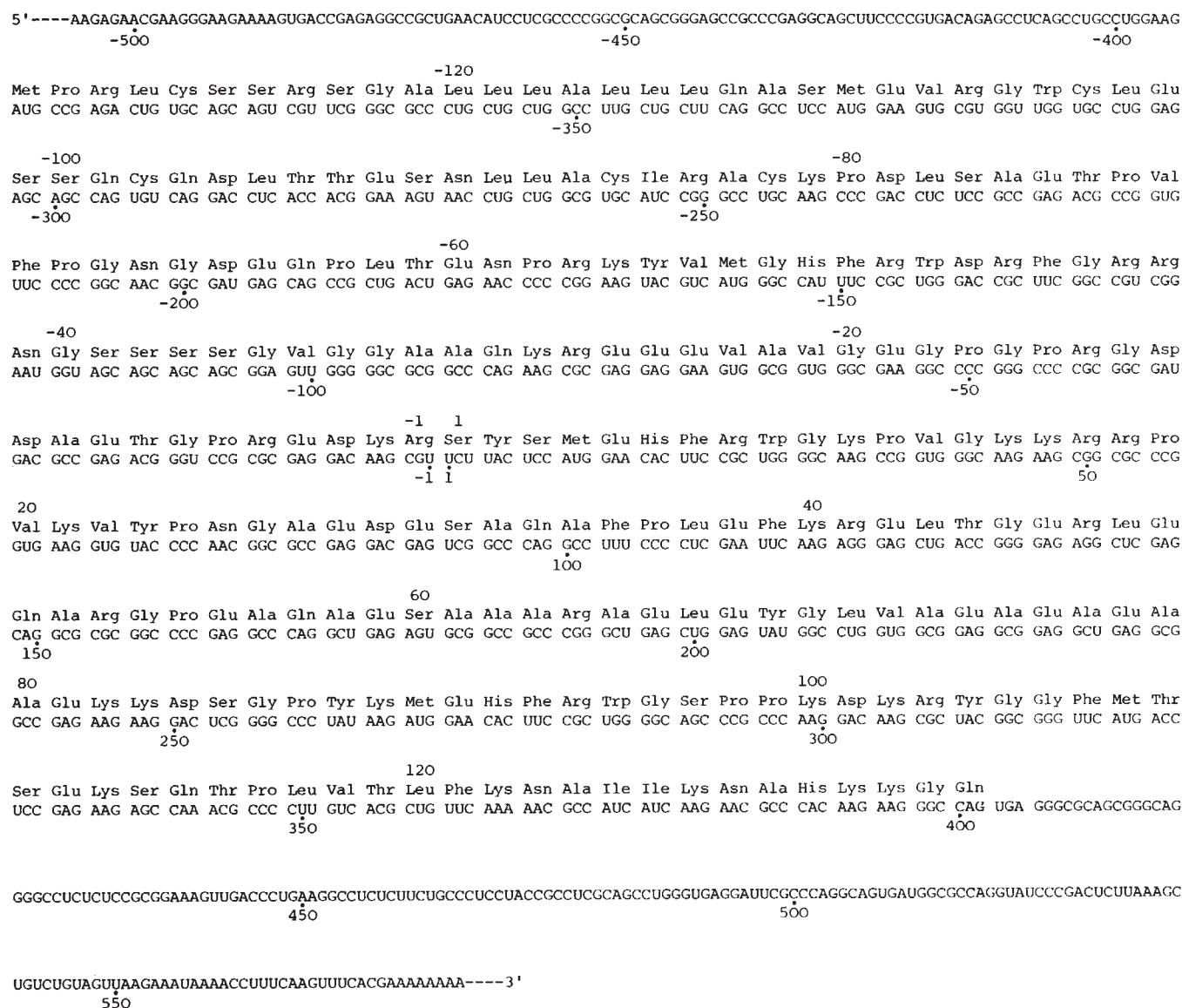


Fig. 2 Primary structure of bovine ACTH- β -LPH precursor mRNA. The nucleotide sequence of the ACTH- β -LPH precursor mRNA was deduced from that of the cDNA insert in pSNAC20. The nucleotide numbers (see legend to Fig. 1) are indicated below the sequence. The predicted amino acid sequence up to the methionine residue encoded by nucleotide residues -393 to -391 is shown above the nucleotide sequence. Amino acid residues are numbered in the direction from amino terminus to carboxyl terminus, beginning with the first residue of ACTH, and the residues on the amino-terminal side of ACTH are indicated by negative numbers; the amino acid numbers are given above the sequence. The sequence of nucleotide residues 1-207 was reported previously¹⁷ and was confirmed by sequencing additional restriction fragments as shown in Fig. 1. The cDNA insert includes a poly(A) tract of eight nucleotides at positions 578-585, but the average length of the poly(A) sequence in the mRNA was previously estimated to be 68 nucleotides¹.

methionine and tryptophan residues at the positions characteristic of the MSH structure and shares with α -MSH serine and glutamic acid residues at equivalent positions. Computer analysis indicates no structural correspondence of other amino acid sequences in the cryptic portion of the precursor with hormones of known sequence³⁹.

From the findings described above, we conclude that the ACTH- β -LPH precursor protein is probably composed of four repetitive structures as shown in Fig. 4. Each unit contains a homologous peptide core segment having an MSH sequence or its analogue, which is followed by or lies within an individually different sequence. Furthermore, each of the repetitive units, as well as the MSH sequences themselves, is flanked on both sides by a pair of basic amino acid residues (or by one of the termini of the precursor molecule). The putative structure of the common precursor protein suggests that each of its units is destined to be separated from its neighbour by proteolytic processing and that further cleavage occurs within the unit to yield smaller peptides.

The peptide fragment consisting of amino acid residues -131 to -58 includes five cysteine residues, and the remainder of the precursor molecule contains no cysteine residue. Other peptide hormones, such as growth hormone and insulin, contain an even number of cysteine residues which form disulphide bonds³⁹. Thus, we can assume that the cysteine residue at position -127 is a constituent of the putative signal peptide, which is removed during the process of secretion, although we cannot exclude the possibility that an odd number of cysteine residues are encoded by the missing portion of the mRNA.

The repetitive nature of the amino acid sequence of the ACTH- β -LPH precursor molecule raises interesting questions concerning both the biological significance of its characteristic structure and the biological functions of possible peptide fragments that may be derived from it. The presence of several partly homologous units within the same molecule suggests that the functions of the component peptides may be related to or coordinated with one another. It is well established that ACTH is essential for the induction and maintenance of glucocorticoid production in adrenocortical cells⁴⁰. However, additional pituitary effects on adrenocortical cells, such as stimulation of mitogenic activity and mineralocorticoid production, cannot be accounted for solely by the action of ACTH⁴⁰. It is an intriguing hypothesis that other peptides which may be elaborated from the precursor molecule are involved in these actions. Another possibility is that these structurally related peptides are involved in neural functions. Several studies have demonstrated that not only endorphins and methionine-enkephalin⁴¹ but also MSHs and related peptides^{42,43} are present in various regions of the central nervous system. In addition to the potent opiate activity of endorphins and methionine-enkephalin⁴⁴, MSH-related peptides have been reported to exert certain effects on brain function, such as learning and retention of new behaviour⁴⁵. Thus, it is attractive to speculate that various peptides derived from the ACTH- β -LPH precursor may act together or antagonistically as modulators of neural functions.

Table 1 Codon usage in bovine ACTH- β -LPH precursor mRNA

Phe	UUU	1	Ser	UCU	1	Tyr	UAU	2	Cys	UGU	1
	UUC	8		UCC	4		UAC	4		UGC	4
Leu	UUA	0		UCA	0	Ter	UAA	0	Ter	UGA	1
	UUG	1		UCG	3		UAG	0	Trp	UGG	4
Leu	CUU	2	Pro	CCU	0	His	CAU	1	Arg	CGU	4
	CUC	4		CCC	11		CAC	3		CGC	10
	CUA	0		CCA	0	Gln	CAA	1		CGA	0
	CUG	13		CCG	7		CAG	9		CGG	5
Ile	AUU	0	Thr	ACU	1	Asn	AAU	1	Ser	AGU	3
	AUC	3		ACC	3		AAC	6		AGC	9
	AUA	0		ACA	0	Lys	AAA	1	Arg	AGA	1
Met	AUG	6		ACG	5		AAG	18		AGG	2
Val	GUU	1	Ala	GCU	3	Asp	GAU	2	Gly	GGU	3
	GUC	2		GCC	16		GAC	8		GGC	17
	GUA	0		GCA	0	Glu	GAA	7		GGA	1
	GUG	8		GCG	8		GAG	22		GGG	5

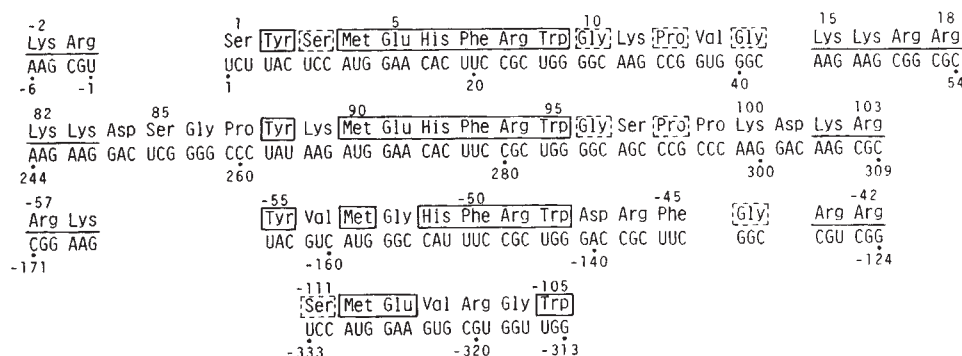
The amino acid sequence of the bovine ACTH- β -LPH precursor has been predicted solely from the nucleotide sequence of the mRNA, assuming that the translational initiation site is located at the methionine residue at position -131. Numbers next to codons indicate the numbers of amino acids using particular codons.

Characteristic features of ACTH- β -LPH precursor mRNA

We have previously observed that the coding regions for ACTH and the first segment of β -LPH contain an unusually large number of repeated nucleotide sequences, suggesting that these regions of the structural gene for the ACTH- β -LPH precursor have evolved by a series of genetic duplications¹⁷. In the present study, this observation has been extended to the remaining portions of the ACTH- β -LPH precursor mRNA. Comparison of the coding regions for α -MSH, β -MSH and γ -MSH strongly supports the view that DNA sequence duplications have occurred during the evolution of these genes (Fig. 3). The nucleotide sequences encoding the common amino acids are conserved, except for a very few silent codon changes; however, those coding for the amino acids that are different in the three peptides are drastically altered. In addition to showing the extensive nucleotide sequence homology just mentioned, computer analysis^{46,47} of the nucleotide sequence data presented in Fig. 2 reveals that the ACTH- β -LPH precursor mRNA contains other segments that seem to represent direct duplications; for example, residues -453 to -443 and 406 to 417; -93 to -82 and 235 to 246; -65 to -54 and 35 to 45; -11 to -2 and 299 to 308; 163 to 187 and 220 to 241; 400 to 411 and 506 to 517. Taken together, these findings support our concept that the structural gene for the ACTH- β -LPH precursor protein has evolved by a series of direct duplications of certain common ancestral DNA segments followed by substitution, addition or deletion of some regions.

Codon choices for the ACTH- β -LPH precursor mRNA have been assigned (Table 1), using the amino acid sequence shown in Fig. 2. No definitive conclusion can be reached concerning codon utilisation because the initiation site for translation has not been established. Nevertheless, codon usage seems to occur nonrandomly, as in the case of other eukaryotic mRNAs²¹⁻²⁵, and specific codon choices seem to be strongly preferred for

Fig. 3 Comparison of nucleotide and amino acid sequences in the regions including MSHs and their analogue. The amino acid sequences are lined up, introducing gaps so as to point out the homologies discussed in the text; for the numbering of nucleotide and amino acid residues, see legends to Figs 1 and 2. The amino acid residues in solid-line boxes occur in at least three of the four structures shown and those in dashed-line boxes are found in two of them. Sequences of two or four consecutive basic amino acid residues are underlined. α -MSH³⁹ represents amino acid residues 1-13, of which the serine residue at position 1 is N-acetylated and the valine residue at position 13 is in the form of an amide; β -MSH³⁹ represents amino acid residues 84-101.



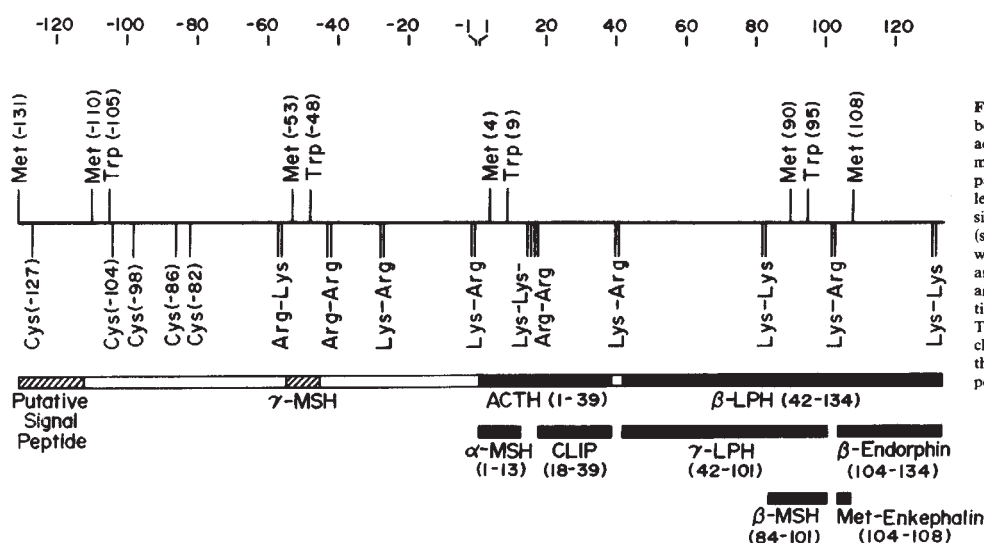


Fig. 4 Schematic representation of the structure of bovine ACTH-β-LPH precursor. Characteristic amino acid residues are shown, and the positions of the methionine, tryptophan and cysteine residues are given in parentheses; for the numbering of amino acid residues, see legend to Fig. 2. The location of the translational initiation site at the methionine residue at position -131 is assumed (see the text). The closed bars represent the regions for which the amino acid sequence was known, and the open and the shaded bars represent the regions for which the amino acid sequence has been predicted from the nucleotide sequence of the ACTH-β-LPH precursor mRNA. The locations of known component peptides are shown by closed bars; the amino acid numbers are given in parentheses. The locations of γ-MSH and the putative signal peptide are indicated by shaded bars; the termini of these peptides are not definitive.

some amino acids. Certain codons (for example, UUC for Phe, CUG for Leu, AAG for Lys) are used repeatedly, whereas other codon choices are either not made or are rare (for example, UUA for Leu, AUA for Ile, GUA for Val). This mode of codon selection reflects an apparent preference for G or C of the third position of mammalian codons²¹⁻²⁴. Assuming that the initiation site is located at the methionine residue at position -131, 116 codons (44%) terminate in G, 112 (42%) in C, 26 (10%) in U and 11 (4%) in A.

Reflecting the selective codon usage, the ACTH-β-LPH precursor mRNA (excluding the poly(A) region) has a high G+C content (65%) which exceeds that of total bovine DNA (39%)⁴⁸. Also, this mRNA exhibits nonrandom dinucleotide frequencies. The abundance of the dinucleotide CG in this mRNA exhibits a marked contrast to its deficiency in other eukaryotic mRNAs²¹⁻²⁵ as well as in total bovine DNA⁴⁹.

The 3' noncoding region of the ACTH-β-LPH precursor mRNA also shares some features with other eukaryotic mRNAs^{21-25,50}. A lengthy inverted repeat (that is, region of two-fold rotational symmetry) exists at nucleotide residues 469-

492 and 521-497, and this inverted repeat follows two in-frame copies of the nonsense codon UGA. Similar sequence features have been reported for the 3' noncoding end of human chorionic somatomammotropin mRNA⁵¹. Also, the sequence AAUAAA (residues 555-560), which is commonly present near the 3' noncoding end of eukaryotic mRNAs, is found in the corresponding region of the ACTH-β-LPH precursor mRNA.

The sequencing data are available from the authors on request.

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Organisation and sequences at the 5' end of a cloned complete ovalbumin gene

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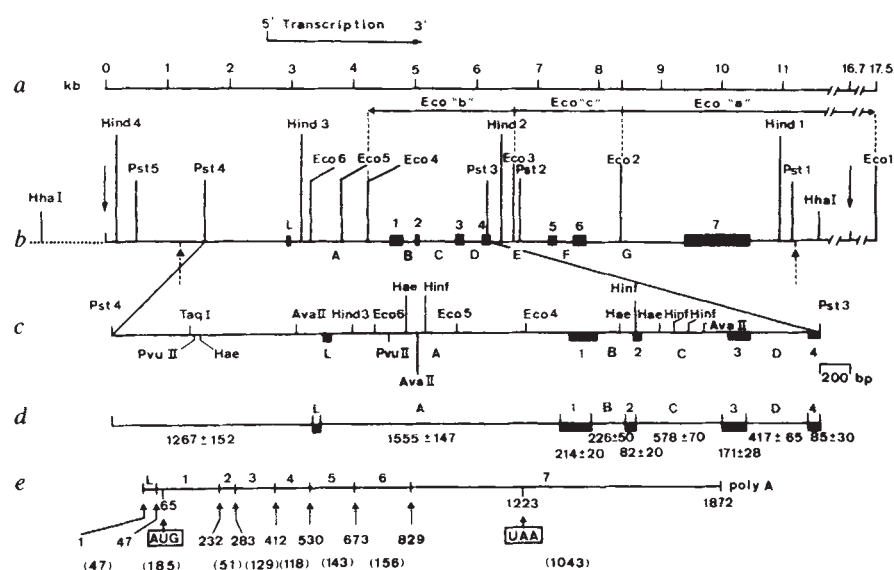
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A clone which contains the complete chicken ovalbumin gene, including its leader coding sequences, has been isolated. From electron microscopic analysis of this DNA we conclude that the minimal size of the transcriptional unit for ovalbumin is 7.7 kilobases. The DNA sequence of the region surrounding the 5' end of the ovalbumin gene is presented. Comparison of this sequence with those of other eukaryotic genes reveals striking similarities, possibly related to a promoter region, approximately 30 base pairs upstream from the site coding for the 5' end of the mRNA.

DUE to molecular cloning, our knowledge of the structure of the chicken ovalbumin gene has advanced rapidly since we first reported that this gene is split¹. The ovalbumin genomic *Eco*RI fragments (*Eco* 'a', *Eco* 'b' and *Eco* 'c', Fig. 1b) have been cloned and analysed²⁻⁵. Seven ovalbumin mRNA (ov-mRNA)

coding regions (exons 1 to 7) are contained in a DNA region of about 6 kilobases and are separated by six introns (B to G) (refs 6-8, and Fig. 1b). A similar conclusion has been reached by Dugaiczky *et al.*⁹. However, the first 45 nucleotides at the 5' end of the 1,872-nucleotide long ov-mRNA^{10,11} are not encoded by sequences contained in exon 1 or in *Eco*RI fragment 'b' upstream from exon 1 (ref. 12). We have called¹² this untranslated sequence of 45 nucleotides the ovalbumin leader, by analogy with the viral terminology. We have assumed that the leader-coding sequences are located upstream from the *Eco*RI site *Eco*4 and are separated from exon 1 by an additional intron A (ref. 12 and Fig. 1b). Thus, the determination of the localisation of the DNA segment containing the leader-coding sequences and its purification are prerequisites for the study of the molecular mechanisms involved in the transcription of the ovalbumin gene and its hormonal control. We report here the isolation of chicken genomic DNA clones which contain a complete ovalbumin gene including the region coding for the ov-mRNA leader. The position of the leader-coding sequences has been determined by electron microscopy and DNA

Fig. 1 Organisation of the ovalbumin gene. *a*, Scale in kilobase pairs for (*b*). *b*, Localisation of the exons (1 to 7, heavy lines) and introns (A to G) of the ovalbumin gene in λ C4-ov5 and λ C4-ov6 within and upstream from the *Eco*RI fragments *Eco* 'a', *Eco* 'b' and *Eco* 'c'. The horizontal dotted line represents λ Charon 4A sequences. The uninterrupted and dashed arrows indicate the limits of the 16.7 and 9.8 kilobase genomic DNA fragments inserted in λ C4-ov5 and λ C4-ov6, respectively. L corresponds to the location of the leader-coding sequences as determined by electron microscopy and DNA sequencing (see text). The *Eco*, *Pst*, *Hind* and *Hha*I sites correspond to *Eco*RI, *Pst*I, *Hind*III and *Hha*I restriction enzyme sites which were mapped in this study or previously (refs 7, 12). *c*, Localisation of *Pvu*II, *Taq*I, *Hae*III (*Hae*), *Ava*II, *Hind*III (*Hind*), *Eco*RI (*Eco*) and *Hinf*I (*Hinf*) restriction enzyme sites within the *Pst*3-*Pst*4 fragment of *b*. L, A, B, C, D, 1, 2, 3 and 4 are as described for *b*. *d*, Schematic representation of the electron microscopic result shown in Fig. 3c and correlation with the restriction enzyme map shown under *c*. The heavy lines L, 1, 2, 3 and 4 represent the RNA-DNA hybrid regions corresponding to the leader-coding regions (L) and to exons 1 to 4, respectively. A, B, C and D correspond to the intronic single-stranded loops A, B, C and D, respectively. The numbers correspond to base pairs or nucleotides and were calculated from measurement of 51 molecules of the type shown in Fig. 3c. *e*, Schematic representation of ov-mRNA. The total length and the position of the AUG and UAA codons are taken from refs 10-12. L refers to the leader sequence and 1 to 7 correspond to the seven domains of ov-mRNA coded by exons 1 to 7. The arrows indicate the limits of these domains, assuming that the splicing events obey the GT-AG rule (see ref. 12 and text). Numbers in parentheses indicate the length (in nucleotides) of the domains.



sequence analysis. The sequences preceding those coding for the 5' end of ov-mRNA have also been determined and compared with those of other genes in a search for possible regulatory signals.

Isolation and mapping of ovalbumin clones containing sequences upstream from exon 1

A chicken erythrocyte DNA library has been constructed by J. Dodgson, D. Engel and R. Axel using a method described by Maniatis *et al.*¹³. Briefly, this approach consists of partially digesting the DNA by *Hae*III and *Alu*I restriction enzymes, selecting DNA fragments which are 15–20 kilobases long, ligating these fragments to the vector λ Charon 4A using *Eco*RI linkers, and infecting *Escherichia coli*. As the recognition sites for both *Hae*III and *Alu*I occur frequently, a partial digest by these enzymes is analogous to random shearing of the DNA. This library was made available to us, with the indication that it contained 400,000 independent clones.

To limit the number of clones analysed to those most likely to contain the 5' region of the ovalbumin gene, we screened² the library using a nick-translated ³²P-labelled *Eco*4–*Hind*2 fragment (see Fig. 1b) as probe. Four clones containing ovalbumin sequences were isolated by screening about 2×10^6 plaques. Two of these (λ C4-ov5 and λ C4-ov6) were shown by preliminary mapping experiments to contain previously uncloned DNA, upstream from *Eco*RI fragment b, and were selected for further analysis.

We have previously shown^{1,7} that the ovalbumin sequences containing exons 1 to 7 are found in two *Hind*III fragments of about 4.6 and 3.2 kilobases (defined by the *Hind*III sites, *Hind*1, *Hind*2 and *Hind*3 in Fig. 1b) and in two major *Pst*I fragments each of about 4.5–4.6 kilobases (defined by *Pst*I sites, *Pst*1–*Pst*2 and *Pst*3–*Pst*4, Fig. 1b). The DNA of λ C4-ov5 and 6 was digested with *Hind*III, transferred to nitrocellulose filters and hybridised with the ³²P-labelled ovalbumin double-stranded cDNA probe (*Hha*ov), as previously described¹. As shown in Fig. 2A (lanes 2 and 5), both clones contain the two *Hind*III fragments. Only one strongly hybridising band, corresponding to the two large *Pst*I fragments, was seen (see legend to Fig. 2A) when the clones were digested with *Pst*I (lanes 3 and 6). From these results we conclude that both clones contain all the DNA between sites *Pst*1 and *Pst*4 (Fig. 1b). In agreement with these results, *Eco*RI fragments 'b' (2.35 kilobases) and 'c' (1.75 kilobases) are present in both clones (Fig. 2A, lanes 1 and 4). However, the 9.2-kilobase fragment *Eco*RI 'a' was replaced in λ C4-ov5 by a 8.4-kilobase band (Fig. 2A, lane 4) and in λ C4-ov6 by a 2.8-kilobase band (lane 1). From these latter results we conclude that the limits of the inserted DNAs at their 3' end are as indicated by arrows at the right-hand side of Fig. 1b.

The DNA of the two clones was digested with *Eco*RI and the fragments stained with ethidium bromide after agarose gel electrophoresis. The results are shown in Fig. 2B. In addition to the two large arms of λ Charon 4A, (bands a and b), fragments of 8.4, 3.2, 2.35, 1.75, 0.5 and 0.45 kilobases were found in the digest of λ C4-ov5 (Fig. 2B, lane 3), whereas fragments of 2.8, 2.5, 2.35, 1.75 and 0.45 kilobases were present in the λ C4-ov6 digest (lane 2). The *Eco*RI fragments of 8.2 (λ C4-ov5), 2.8 (λ C4-ov6), 2.35 and 1.75 kilobases have already been mapped (see above and Fig. 1b). The above results indicate that the other *Eco*RI fragments are necessarily located on the 5' side of the *Eco*4 site. Their relative positions in the λ C4-ov5 '*Pst*3–*Pst*4' fragment (Fig. 1c) which was sub-cloned in pBR 322, were derived by further mapping with *Taq*I, *Pvu*II, *Hae*III, *Ava*II, *Hinf*I, *Hind*III and *Eco*RI enzymes (not shown). We found that the *Pst*3–*Pst*4 fragment contains, in addition to the already known *Eco*4 site, two *Eco*RI sites, *Eco*5 and *Eco*6, at 0.45 and 0.95 kilobases from *Eco*4, respectively (Fig. 1c). Therefore, there are 3.2 kilobases between *Eco*6 to the 5' end of the inserted DNA (Fig. 1b, arrow) to which the *Eco*RI linker was added. Parallel experiments showed that the *Eco*RI site *Eco*6 is missing in the DNA fragment inserted in clone λ C4-ov6, which

probably corresponds to an additional allelic form of the ovalbumin gene^{3–5,7,9}. From the size of the *Eco*RI fragments of clone λ C4-ov6, we conclude that the 5' end of the inserted DNA is 2.5 kilobases from *Eco*5 (Fig. 1b, dashed arrow).

These analyses indicate that the sizes of the chicken DNA fragments inserted in clones λ C4-ov5 and 6 are 16.7 and 9.8 kilobases, respectively (Fig. 1b). In λ C4-ov5 there are about 10.3 kilobases separating the 5' end of the inserted fragment from the extremity of exon 7, which codes for the 3' end of ov-mRNA. The corresponding distance is 9.2 kilobases in clone λ C4-ov6. Considering these sizes, we expected that these clones should contain the sequences coding for the ov-mRNA leader.

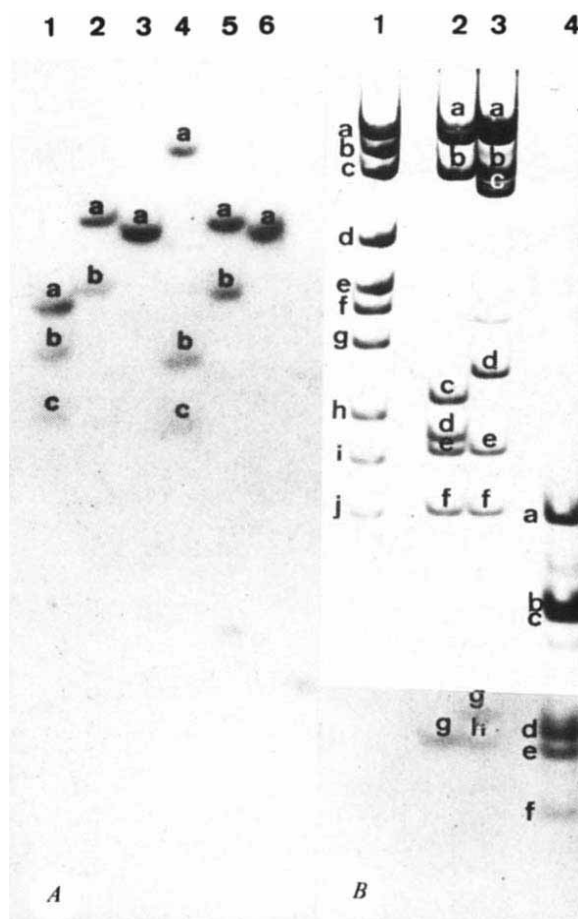
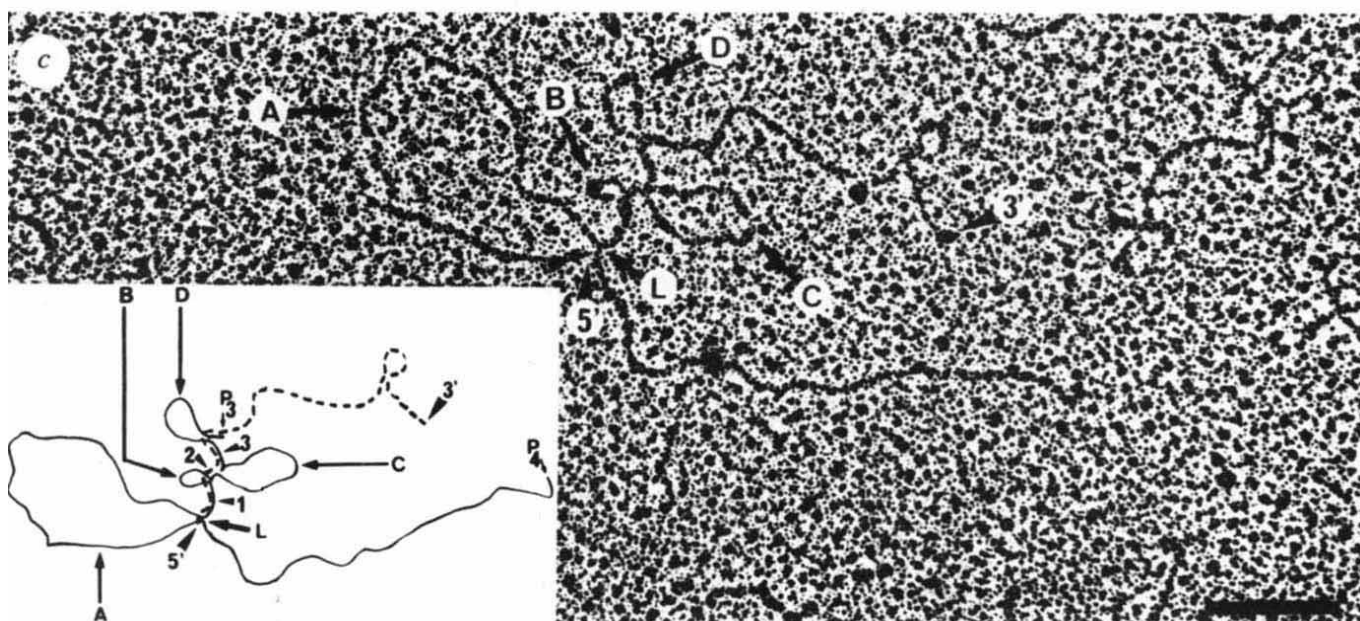
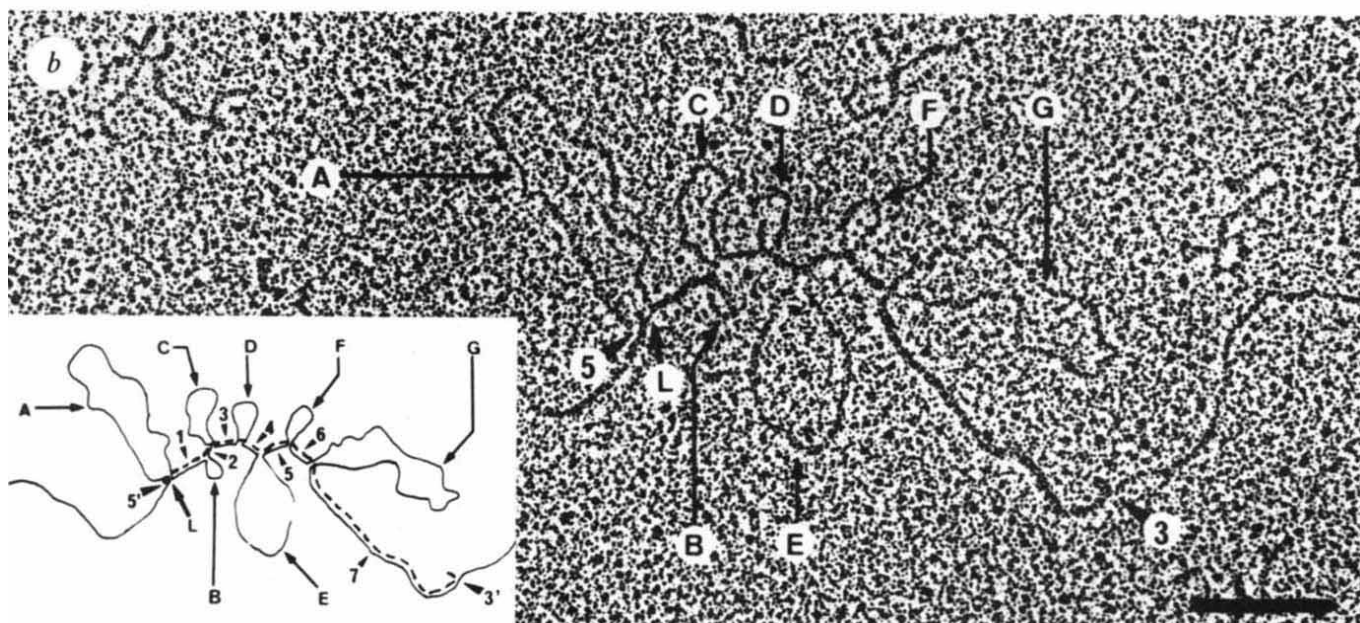
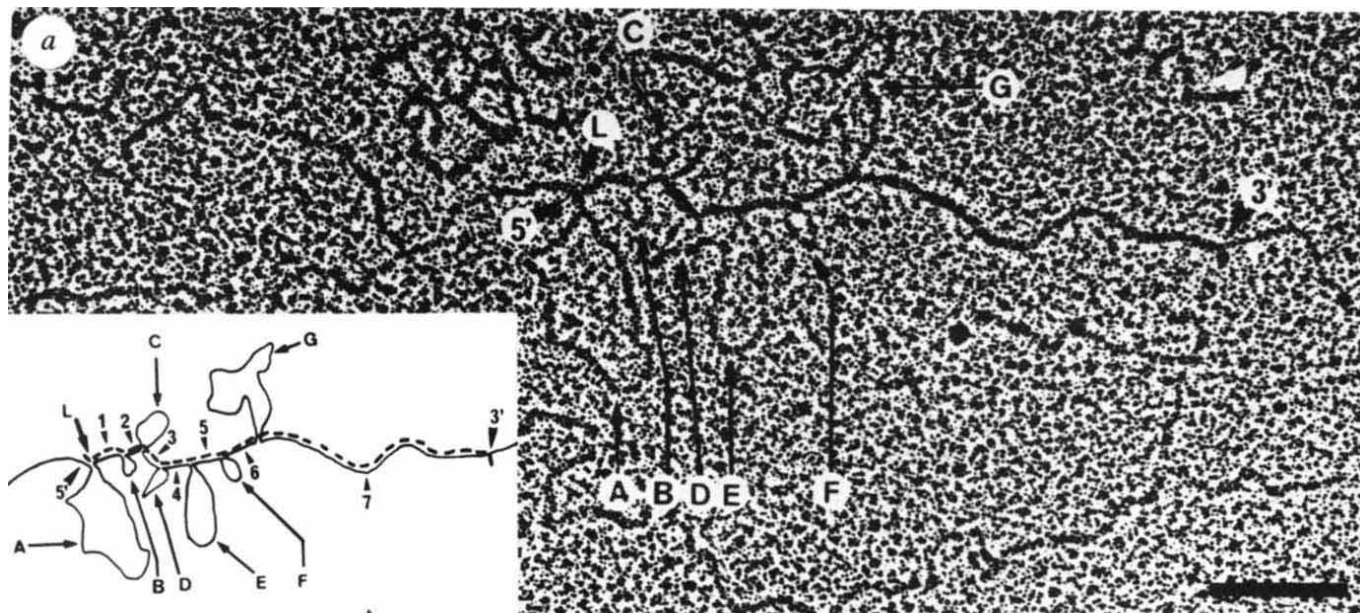


Fig. 2 A, Detection of restriction enzyme fragments which hybridise to the double-stranded ovalbumin cDNA probe ³²P-*Hha*ov (ref. 1), in *Eco*RI, *Pst*I and *Hind*III digests of λ C4-ov5 and λ C4-ov6 DNAs. The DNA was digested, electrophoresed on a 1% agarose gel, transferred onto nitrocellulose filters, hybridised with a ³²P-*Hha*ov probe and revealed by autoradiography as previously described¹. Lanes 1, 2 and 3: *Eco*RI, *Hind*III and *Pst*I digests of λ C4-ov6 DNA, respectively. Lanes 4, 5 and 6: *Eco*RI, *Hind*III and *Pst*I digests of λ C4-ov5 DNA, respectively. Sizes of the hybridising fragments (in kilobases): lane 1, 2.8 (a), 2.35 (b) and 1.75 (c); lanes 2 and 5, 4.5 (a) and 3.2 (b); lanes 3 and 6, 4.5–4.6 (a) (the two fragments of 4.55 and 4.4 kilobases, which were clearly seen on the same gel after ethidium bromide staining, were poorly resolved on the autoradiogram); lane 4, 8.4 (a), 2.35 (b) and 1.75 (c). B, Detection of *Eco*RI restriction enzyme fragments of λ C4-ov5 and λ C4-ov6 DNAs by staining with ethidium bromide after electrophoresis on a 0.8% agarose gel. Lane 1, *Bam*HI and *Eco*RI restriction enzyme fragments of adenovirus-2 DNA used as size markers (in kilobases): 21.05 (a), 14.6 (b), 10.4 (c), 6.3 (d), 4.7 (e), 4.4 (f), 3.71 (g), 2.9 (h), 2.05 (i) and 1.87 (j). Lane 2, *Eco*RI digest of λ C4-ov6 DNA; size of the fragments (in kilobases): 19 (a), 12 (b), 2.8 (c), 2.5 (d), 2.35 (e), 1.75 (f) and 0.45 (g). Lane 3, *Eco*RI digest of λ C4-ov5 DNA; size of the fragments: 19 (a), 12 (b), 8.4 (c), 3.2 (d), 2.35 (e), 1.75 (f), 0.55 (g) and 0.45 (h). Lane 4, SV40 DNA fragments of a partial *Hind*III digest used as size markers (in kilobases): 1.77 (a), 1.15 (b), 1.1 (c), 0.52 (d), 0.45 (e) and 0.22 (f). To show clearly bands g and h (lanes 2 and 3) the lower part of the figure was exposed for a longer time.



possible to sequence the mRNA directly from its 5' end¹⁷. The technique used by McReynolds *et al.*¹⁰ and by O'Hare *et al.*¹¹ to establish the mRNA sequence shown in Fig. 5 involves elongation of a DNA primer hybridised to the 5' end region of the messenger with reverse transcriptase in the presence of chain-terminating dideoxynucleotides. This technique could lead to premature termination if important secondary structure exists in the RNA.

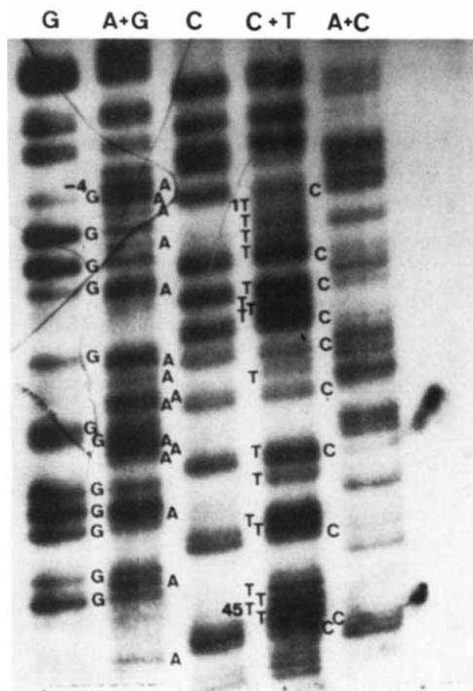


Fig. 6 Autoradiogram of sequencing gel showing the leader sequence. The sequence was read from the strand coding for ov-mRNA. An *HphI/HaeIII* fragment (Fig. 4) ³²P-labelled¹² at the *HphI* end was subjected to five base-specific cleavage reactions (G, A>G, C+T, C, A>C) according to the procedure of Maxam and Gilbert¹⁶. The products were analysed on a denaturing gel according to Sanger and Coulson⁴⁵. The numbers (45, 1, -4) indicate the position of the nucleotides corresponding to those shown on the non-coding strand in Fig. 5.

Sequences at the splice points

As shown in Fig. 5, the DNA sequences coding for the 5' end of ov-mRNA are interrupted at a position corresponding to nucleotide 47 of the messenger. This result agrees with our previous work which established that exon 1 does not code for the first 45 nucleotides of the mRNA¹². Nucleotides 46 and 47 (AG) of the messenger could be encoded either by the leader-coding region or by exon 1, due to the presence of the repetition of the dinucleotide AG at both ends of intron A. As previously discussed for the other ovalbumin introns¹², this prevents the definition of a unique splicing point. We have proposed that all intron-exon junctions are closely related and fit the general pattern 5'-TCAGGTA-3' (non-coding strand) at the 5' end of an intron, and 5'-TXCAGG-3' at the 3' end of the same intron¹². Others have stressed this similarity¹⁸⁻²⁰. The additional pair of junctions that we describe here is consistent with this proposal. Moreover, our present results also agree with the 'GT-AG rule' (ref. 12) which allows one to propose unique excision-ligation points common to all introns, despite the existence of the direct repeats. These points are indicated in Fig. 5 by the vertical dotted lines, with dinucleotides GT and AG at the 5' and 3' ends of intron A, respectively. Finally, as for other introns^{12,21}, intron A contains a pyrimidine-rich stretch at its 3' end on the non-coding strand.

The ovalbumin transcription unit

Assuming that no nucleotides were missed when sequencing the 5' end of ov-mRNA, our present studies establish that about 7.7 kilobases separate the genome regions coding for the 5' and 3' extremities of ovalbumin mRNA. Recent *in vivo*²² and *in vitro*²³ studies on primary transcripts from the major late transcription unit of adenovirus-2 strongly suggest that the cap site and the transcription initiation site are coincident and that the initiating residues of the primary transcript are conserved in mRNA as the capped 5' terminus. If, as for adenovirus-2, the ov-mRNA capped sequence identifies the site for the initiation of transcription, the minimal size of the ovalbumin transcription unit should be about 7.7 kilobases. A complete co-linear RNA transcript should then be in the range of 7,700 nucleotides, about four times the size of the mature mRNA. In fact, there is evidence for an ovalbumin gene transcript of approximately this size^{24,25}. Although there is no convincing evidence from these latter studies for ovalbumin RNA molecules longer than this transcript (our previous determination^{8,12} was overestimated due to a problem at that time with RNA size markers of high molecular weights), we cannot exclude the possibility that some processing has already occurred and that the ovalbumin transcription unit is longer than 7.7 kilobases.

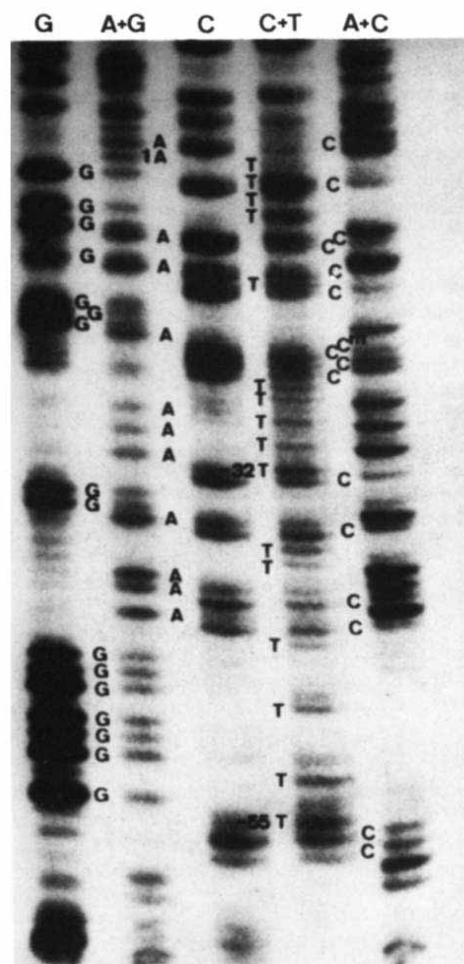


Fig. 7 Autoradiogram of sequencing gel showing the sequence upstream from the leader sequence. The sequence was read from the non-coding strand. An *AvaII/HindIII* fragment (Fig. 4) ³²P-labelled at the *AvaII* end¹², was sequenced as described in the legend to Fig. 6. The numbers (-55, -32, 1) correspond to the positions of the nucleotides of the non-coding strand as shown in Fig. 5. Cm corresponds to the position of a methyl C which belongs to an *EcoRII* site (G was unambiguously found at this position when the coding strand was sequenced).

Possible control regions

It has been suggested that complementarity between a purine-rich sequence, 3'-UAGGAAGGCGU-5', present at the 3' end of 18S ribosomal RNA and sequences of the 5' non-coding regions of many eukaryotic mRNAs might contribute to mRNA recognition by ribosomes²⁶. As previously noted²⁷, a sequence of nine nucleotides complementary (with one mismatch) to this 18S RNA region is found between positions 25 and 33 of the ovalbumin mRNA (Fig. 5, underlined by dotted line). This sequence is immediately preceded by a region (nucleotides 3–22) which can form a base-paired loop¹⁰. There is no such extensive complementarity with the 3' end of 18S rRNA in the messenger nucleotide sequence located between position 48 and the AUG initiation codon (position 65) and encoded for by exon 1. One can therefore speculate that one of the functions of splicing in the 5' non-coding region would be to transpose such a sequence to the vicinity of the initiation codon. A similar translocation has been described for the adenovirus tripartite leader^{22,28}.

A promoter is functionally defined in prokaryotes as the region of the DNA which precedes a transcription unit and indicates the startpoint for RNA polymerase. Most sequenced prokaryotic promoters have two common interacting sites, the Pribnow box²⁹ and the recognition site³⁰ (for other refs, see refs 31–33). The Pribnow box, which is of the form 5'-TATAATG-3', precedes the transcription-initiation site by five to seven nucleotides and belongs to the model sequence (ref. 32)



where the upper and lower case letters correspond to bases present at high and moderate frequency, respectively (the lower line shows the bases which are most often found as alternatives). The recognition site is a region centred at about 32 base pairs before the beginning of the mRNA and has a structure clearly related to the sequence 5'-TGTTGACATTT-3' (refs 30, 32, 33). Assuming that the ovalbumin mRNA cap site and the startpoint for transcription are coincident, sites analogous to the prokaryotic common interacting promoter sites could be contained within the region upstream from the leader-coding sequence. In a search for such sequences, we have compared the DNA sequences upstream from the cap site for ovalbumin, rabbit β -globin (J. Van der Berg *et al.*; A. Efstratiadis, T. Maniatis and L. Lacy; personal communications), mouse β -globin major³⁴ and adenovirus-2 major late²² genes. When these sequences are aligned so as to emphasise sequence homologies as shown in Fig. 8, there is clearly a 7-base pair AT-rich region of approximate homology centred at about 28 base pairs from the initial nucleotide of the mRNAs. This region of homology, which may be a common interacting promoter site, is very similar to the 'Hogness box' which is of the form 5'-TATAAATA-3' (D. Hogness, personal communication) and has been found in an analogous location upstream from the startpoint of transcription of several genes including the histone genes^{35–37}. No similar sequence exists in analogous positions upstream from the 5' end of any of the other ovalbumin exons (ref. 12, and our unpublished results). Note that, in all four cases compared, the AT-rich region of homology is flanked by GC-rich segments (Fig. 8), suggesting that the 'Hogness box' could be extended by three or four residues. There is some resemblance between this eukaryotic region of homology and the prokaryotic model sequence which has the Pribnow sequence at one end (Fig. 8), suggesting that prokaryotic and eukaryotic promoters could have some common features. However, the 'Hogness box' is in a position more closely resembling that of the prokaryotic recognition site than that of the Pribnow box. In this respect, it is interesting that the recognition site of the *E. coli lac* promoter centred at about -30 is bounded on both sides by GC-rich sequences³⁸.

There is a striking similarity between the 10 residues which precede the first nucleotide of adenovirus-2 late major and

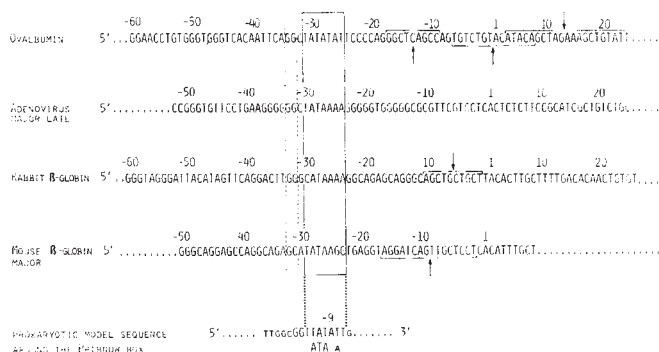


Fig. 8 Comparison between DNA sequences of ovalbumin, rabbit β -globin, mouse β -globin major³⁴ and adenovirus-2 major late²² genes upstream from the 5' end of the mRNAs. The sequences are aligned to show their similarities. Numbering is in base pairs and negative numbers are given to nucleotides upstream from the 5' end of the mRNAs (position 1). The AT-rich region of homology is boxed with a continuous line. The GC-rich region of homology is boxed with a dashed line. Lines above or below the sequences delineate regions of partial two-fold rotational symmetry. Centres of symmetry are indicated by arrows. The prokaryotic promoter model sequence³² which has the Pribnow box at one end is given for comparison (the upper and lower case letters correspond to bases present at high and moderate frequency, respectively).

mouse β -globin major mRNAs, as previously noted by Ziff and Evans²². However, this homology does not extend to the other genes included in Fig. 8. Additional sequence data are required to decide whether there is a second, but weaker, region of homology with a location similar to that of the Pribnow box.

Regions of partial (hyphenated) two-fold symmetry are found around the probable start of transcription of the ovalbumin gene downstream from the possible RNA polymerase interacting site centred at about -28 (Fig. 8). Regions of hyphenated two-fold symmetry are also present in a similar location in the rabbit and mouse β -globin genes, but are absent in the case of adenovirus (Fig. 8). Elements of complete or partial two-fold rotational symmetry are found in prokaryotic operators and at other sites controlling prokaryotic transcription (refs 30, 32, 38–43). Further studies will reveal whether specific regulatory proteins, possibly related to the steroid hormone induction system, could bind to these symmetrical sequences, which surround the probable ovalbumin transcription initiation site.

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letters

GX339–4: a new black hole candidate

THE large area survey instrument on HEAO 1 has observed that the galactic X-ray source GX339–4 (4U1658–48) varies on time scales from tens of milliseconds to hundreds of days. Its long term variability was known from measurements made by OSO 7¹ and by Uhuru² but this is the first observation of aperiodic variability on time scales of tens of milliseconds to a few seconds. We show here that the overall characteristics of GX339–4 place it in the same class of X-ray sources as Cyg X–1 (ref. 3 summarises Cyg X–1) and Cir X–1^{2,4–6}.

The HEAO observations were made on 10–14 September 1977, 8–12 March 1978 and 10–14 September 1978. The average intensities in the 1–20 keV range of GX339–4 during these observations were ~ 0.25 , ~ 0.01 , and ~ 0.8 ct cm^{–2} s^{–1}, respectively (1 HEAO ct cm^{–2} s^{–1} $\equiv$ 200 Uhuru flux units).

These values are approximate because the source was highly variable during the September 1977 observation, and because only a few quick-look observations were used to determine the March 1978 and September 1978 intensities. The first two intensities correspond to the 'low' and 'off' states defined by OSO 7¹. This is the first observation of detectable emission during the 'off' state. The third intensity falls between the 'low' and 'high' state values.

Detailed analysis has been made of ~ 200 s of data from the 'low' state observation in September 1977. GX339–4 was observed 20 times, ~ 10 s each, as the HEAO collimators ($1^\circ \times 4^\circ$ FWHM) scanned across the source. Fifteen of these scans were made with a time resolution of 320 ms and five were made with a resolution of 5 ms.

Figure 1 shows the intensity profile of GX339–4 observed with 320 ms resolution during three scans separated in time by 10 h and 18 h. The source intensities shown have been corrected

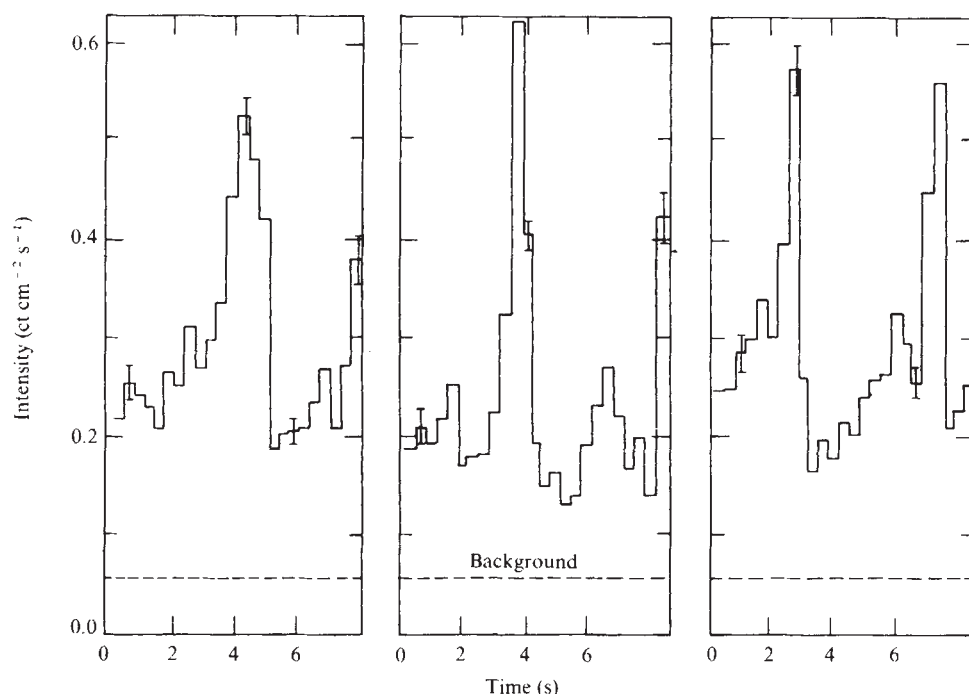


Fig. 1 Three scans through GX339–4 at 0.32 s resolution. Source intensities are plotted corrected for collimator response and with background subtracted. The background levels are shown for comparison.

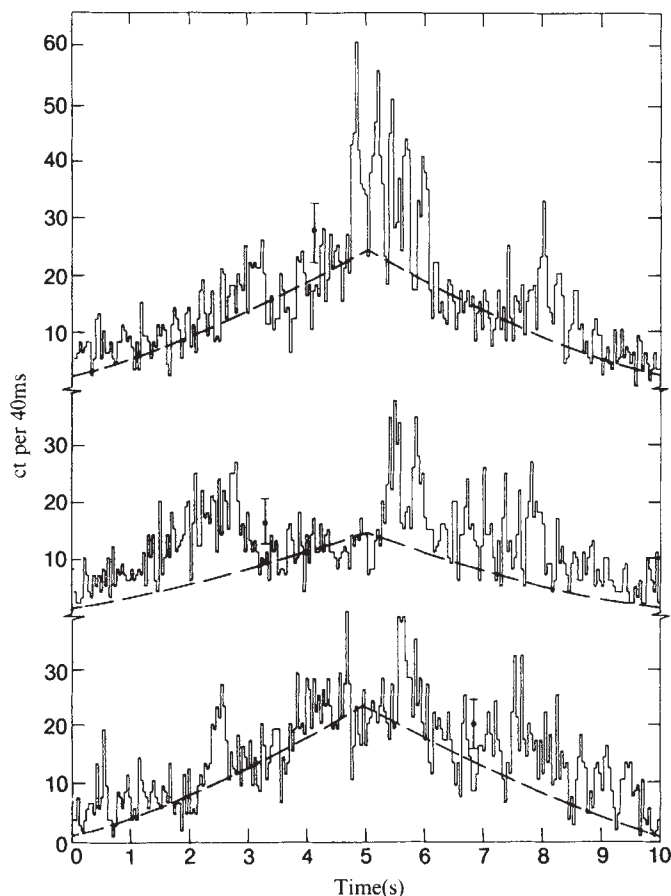


Fig. 2 Three scans through GX339-4 plotted at a resolution of 40 ms. No corrections have been made to the rates. The dashed lines show the collimator's response.

for collimator response and the background has been subtracted. The background (cosmic diffuse X-ray and particle) was determined from measurements before and after the source passed through the field of view. Flaring activity with changes in intensity by factors of 3 on time scales of 0.32 to 1.28 s is evident. A study of the ~ 150 s of data from the source indicates that these flares occur at a rate of $\sim 0.3 \text{ s}^{-1}$, have an average duration of ~ 1 s and contain about 15% of the power emitted by the source. The intensity of the source averaged over 10 s is variable of a factor of ~ 2 on 5-h time scales.

Figure 2 shows three scans through GX339-4 with a resolution of 40 ms. The rates have not been corrected for collimator response; the dashed lines show the expected response of the collimator to a non-varying source. Flares exhibiting intensity changes of a factor of ~ 3 with rise times from 40 ms to 160 ms are evident. Of particular interest is the series of five sequential flares occurring in the top scan with separations of ~ 250 ms (the separation between the peaks of the first and second flares is ~ 350 ms). This type of sporadic quasi-periodic behaviour is similar to episodes observed in Cyg X-1³ and Cir X-1⁶, and will require more detailed investigation to assess its significance.

The time scales of the variability of GX339-4 are displayed in Fig. 3. This figure was obtained by grouping 40 s of high-resolution data into non-overlapping segments covering 1.6 s, 0.8 s, 0.4 s and 0.05 s. For each of these segments a value of χ^2 was calculated using 10 resolution elements (durations of 160, 80, 40 and 5 ms, respectively) within each grouping. Plotted are the normalised number of segments with $\chi^2/9$ (nine degrees of freedom) greater than the value given by the abscissa. The total number of segments used range from 20 for the 160 ms grouping, to 800 for the 50 ms grouping. The calculated distribution for random data is shown for comparison. This analysis indicates that the source is not significantly variable on time scales between 5 and 50 ms, but is highly variable on longer time scales. HEAO 1 data from Cyg X-1 analysed in the same manner exhibit similar variability⁷. The true characterisation of the variability of GX339-4 will require a larger data base. Unfortunately, the source was in its 'off' state during a pointed observation with HEAO 1 performed on 2 October 1978. Data from a second pointed observation have not yet been analysed.

Fourier analysis of the limited data base failed to reveal any significant periodic power from 10 ms to 2.5 s. In contrast, an autocorrelation analysis of the data reveals significant correlation times from 0.3 to 0.6 s. This suggests that a shot noise model, similar to that used for Cyg X-1⁸, may be the best way to characterise its variability.

The temporal characteristics of GX339-4 resemble those of Cyg X-1^{3,7,8} and Cir X-1^{4,6}, two X-ray sources which are candidates for black holes in a binary system. These characteristics are summarised in Table 1. Much of the information in Table 1 is contained in ref. 9. Cir X-1 and Cyg X-1 are known to be binaries, whereas the data on GX339-4 are insufficient to reveal a binary nature. With the optical counterpart of GX339-4 now identified^{10,11}, this information may soon become available. All three sources have X-ray spectra which are harder during their low emission states. In a study of 43 galactic sources with Uhuru¹² only Cir X-1 and Cyg X-1 exhibited this extreme bimodal spectrum. Their soft-mode spectra are the second and third softest in the sample, the softest of all being GX339-4. However, GX339-4 did not exhibit striking variability in the Uhuru data. Cir X-1 and GX339-4 have what

Table 1 Characteristics of black hole candidates

	Cyg X-1	Cir X-1	GX339-4	V861 Sco OAO1653-40
1. Variability				
Orbital period	5.60 d	16.6 d	—	7.85 d
Bimodal X-ray spectrum	Yes	Yes	Yes	Unknown
Extended 'off' states	No	Yes	Yes	Unknown
Correlation time	0.5 s	Variable	0.3-0.6 s	Unknown
Shortest time scale	≥ 1 ms	≥ 1 ms	≥ 10 ms	Unknown
2. Optical companion/ emission lines	HD226868 O9.7 Iab $m_V = 8.9$	Faint highly reddened $m_R = 16$ strong H α and HeI emission	$m_V = 16.6$	HD152667 = V861 Sco B0.5Ia H α emission
3. Mass function	0.22 $M_\odot$	—	—	0.487 $M_\odot$
M_x	$\geq 10 M_\odot$	—	—	7-11 $M_\odot$
4. Distance	~ 2.5 kpc	$\geq 8 \text{ kpc}^{14}$	≥ 4 kpc	2 kpc^{15}
5. Maximum luminosity (erg s^{-1})	$\sim 2 \times 10^{37}$	$\geq 10^{38}$	$\geq 2 \times 10^{37}$	1.5×10^{35}
6. Other emission	Radio, IR	Radio	—	UV

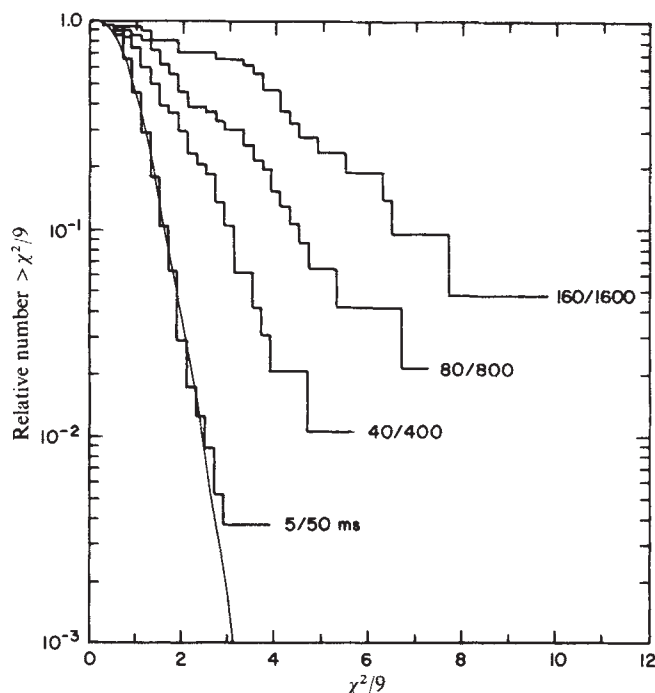


Fig. 3 The integral χ^2 distribution for corrected data sampled from GX339-4 on different time scales. The solid curve shows what is expected for random data. The numbers adjacent to the histograms give the range in time over which variability was tested (for example, the range 5–50 ms is indicated as 5/50). See text for details.

can be characterised as an 'off' state. We find that GX339-4 has an intensity of $\sim 0.01 \text{ ct cm}^{-2} \text{ s}^{-1}$ (~ 2 Uhuru flux units) while in this state. Cyg X-1 is not known to have a similar 'off' state. All three sources exhibit variability on time scales ≤ 100 ms.

Of the first three sources listed in Table 1, Cyg X-1 is the best black hole candidate. Its variable X-ray emission characterises it as a compact object. Furthermore, it is in a binary system for which both the orbital period and velocity are known³. From either the absence of eclipses or from photometric observations of the optical primary, it is possible to place a lower mass limit on the X-ray secondary. This is above the theoretical limit for neutron stars.

A complementary line of argument leading to a black hole interpretation of Cyg X-1 is derived from its variability over such a wide dynamic range of time scales, from milliseconds to years. No persistent periodic pattern has been observed; this is consistent with the central object being different from a pulsing neutron star. Other models have been suggested³ but none seems more plausible than the black-hole model.

The case for regarding Cir X-1, and now GX339-4, as black holes rests on the similarity of their X-ray emission to that of Cyg X-1. They exhibit a similar range of time scales, essentially the same luminosity, and the same bi-modal spectral behaviour.

Table 1 includes the characteristics of V861 Sco which has been recently identified as an X-ray source¹³. The large mass of the non-optical companion and its compact nature, as inferred from the X-ray eclipses when it is occulted by the primary star, are strong evidence in favour of its identification as a black hole. Its luminosity is a factor of 100 less than the other candidates listed. This may be indicative of a fundamental difference in their natures. The determination of the characteristics of the variability of V861 Sco awaits future higher sensitivity studies in progress by satellites such as HEAO and SAS 3.

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Semi-periodic pulsations in circinus X-1

CIRCINUS X-1 (4U1516-56) was first detected in 1969 (ref. 1) and has since been studied in X-ray, radio, and optical wavelengths. Particular interest in this source has resulted from its resemblance to Cygnus X-1—a suspected black hole. Both objects have X-ray luminosities in the range 10^{37} – $10^{38} \text{ erg s}^{-1}$ (refs 2,3). Both exhibit rapid intensity variations with timescales as low as 1 ms (refs 4,5), and as long as seconds or days^{6–8}. Both X-ray sources are in binary systems; the orbital period reported for Cir X-1 is 16.585 d (ref. 9). Cir X-1 differs from Cyg X-1 in that the total mass of the binary pair is unknown. While an optical candidate has been found¹⁰, no estimation of its mass has been made, so that the evidence for its being a black hole is not as strong for Cir X-1. Variable IR emission¹¹ has been reported from the companion. There have been conflicting reports of X-ray periods of the order of seconds: Margon *et al.*¹ suspected a period of 0.685 s and Toor reported a possible 2.2 s period⁴. We report here our investigations, using HEAO A-1, of these semi-periodic pulsations in Cir X-1.

The main portion of the HEAO A-1 experiment consists of four large-area xenon-methane counters with a combined area of 6,800 cm^2 and an effective energy range of 0.5–25 keV. The counters have a $1^\circ \times 4^\circ$ collimator. The roll of the HEAO A-1 causes a point source to be scanned by these modules in 10 s, and a source remains in view for approximately 8 d. The scan modules are on the -y side of the spacecraft. On the opposite side is module 7, which has 1,900 cm^2 effective area. Its collimator, which is $8^\circ \times 2^\circ$, has the longer direction aligned with the scan, so that a source remains in view for about 80 s. Timing resolution is normally 320 ms for all modules, but 5 ms resolution is available.

Before 0200 UT on 28 August 1977, the observed intensity of Cir X-1 was low. At that time, there was a dramatic increase and the source continued in a high state for two more days, after which it left the field of view. Throughout this time the average intensity varied by as much as a factor of 2 during the 15–30-min intervals between successive scans.

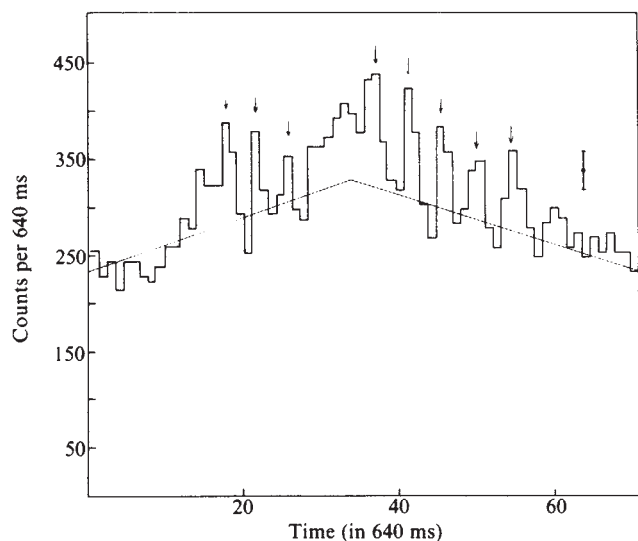


Fig. 1 A scan through Cir X-1 with module 7 using 640-ms sums. The triangular response of the collimators is represented by the two straight lines. A steady source output should follow these lines, but regularly spaced pulses appeared instead. The spacing seemed to be 2.5 s (there are 10 pulses in 39 bins, that is, the period is 2.50 ± 0.06 s).

The data base accumulated during the high state consists of 77 scans with modules 1-4, and eight good scans with module 7. Five of the scans with modules 1-4 used 5-ms timing resolution, but all of the module 7 scans had 320-ms timing resolution. The total observing time from all scans is 20 min.

Erratic intensity variations were observed on all scans, and on many scans no pattern in the variability was evident. A notable exception was the pass shown in Fig. 1, where a train of pulses lasting for 40 s was seen by module 7. The time between successive pulses was 2.5 s. On several scans, modules 1-4 observed similar pulsing. Figure 2 shows two scans, one with the pulsing and one without.

Although not all scans showed periodic pulsations, the pattern on the scan shown in Fig. 1 was sufficiently distinct (with individual pulses standing out at 5σ) for a more systematic search for periodicity to be undertaken.

In this search, four hypotheses were considered: (1) the pulsing in Fig. 1 was of instrumental origin; (2) Cir X-1 varies periodically with a 2.5 s period; (3) the source is not strictly periodic, but has some quasi-periodicity, such as a characteristic recovery interval following each burst or perhaps a periodic component which is sometimes clearly present and other times absent or masked by aperiodic variability; and (4) the intensity variations are aperiodic, that is, burst times are completely random (shot noise).

To test the third conjecture required high sensitivity, so that individual bursts could be isolated and their times could be determined.

Module 7 has intermittently exhibited a high, irregular count rate, thought to be due to electrical noise. At other times it responds normally to sources and has a low background rate. We have devised four data acceptance criteria: first, the count rate before and after a source must reach the normal background level; second, the count rate before and after a source must follow Poisson statistics; third, response to nearby constant sources must follow the known collimator response function; and finally response to Cir X-1 must not extend beyond the full width of the collimator. Eight scans of Cir X-1 on module 7 fulfilled these criteria. As we do not fully understand the nature of the malfunction, it is still possible that the periodicity in Fig. 2 is an instrumental effect. It should be kept in mind that scans from modules 1-4, which are free of this problem, show the same quasi-periodic pulses (Fig. 2).

The search for periodic pulsations was conducted using three methods. (1) Fourier analysis. Each of the scans from module 7 was Fourier analysed. Only one showed a significant peak at 2.5 s. The expectation value for this peak was 1 in 220. (To check for systematic effects, module 7 data at different times was Fourier analysed and no significant peak at 2.5 s was found). Scans from modules 1-4 were not Fourier analysed, there being only 32 points per scan, against 320 in module 7. (2) Epoch folding. The epoch-folding technique is more sensitive than Fourier analysis and can be used when the period is approximately known. Again, only the single pass from module 7 showed a significant peak. (3) Epoch folding of multiple passes. Data can be epoch folded from several scans of the source by inserting strings of zeros to fill the half-hour intervals between scans. A certain assumption needs to be made about unknown orbital parameters of the binary system which was that these effects were negligible. A search for periods near 2.5 s with this method failed to find a significant periodicity.

A comparative test of the hypotheses of quasi-periodicity and random bursting was made using the distribution of intervals between successive bursts. A rise in count was called a burst only if it stood out by at least 2σ above immediately adjacent data, that is, when there was a bin with high count which was both preceded and followed by bins with a count at least 2σ lower. The time of the burst was identified with the local maximum, and the inter-burst interval was the difference in time between two successive bursts. Figure 3 shows the distribution of such intervals.

If the bursts are randomly distributed in time, the distribution of intervals will be exponential, with a characteristic time which is the inverse of the mean rate. The circles show the distribution

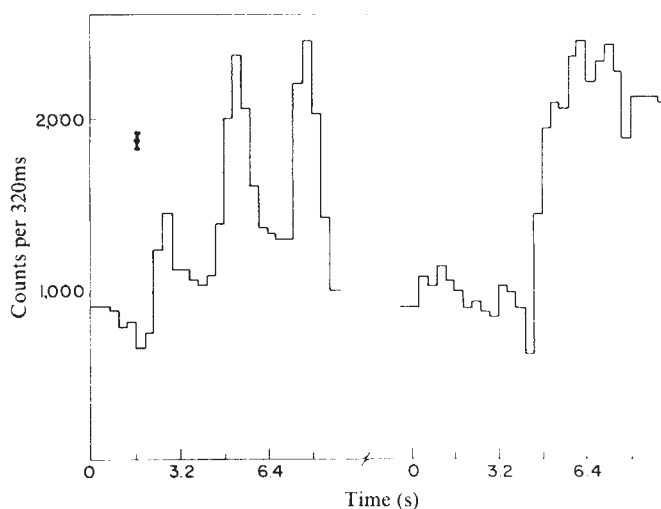


Fig. 2 Two consecutive scans from module 1-4 (corrected for collimator response). On the left one can see a train of three 2.5-s pulses; on the right, the normal erratic behaviour of Cir X-1.

expected if we fit the characteristic interval to the observed rate of bursting. There is a large excess in the number of intervals falling near 8 bins, or 2.5 s. The χ^2 value for the observed distribution is 57, giving a probability of 10^{-5} . The poor fit is due primarily to the contribution from intervals near 2.5 s. We therefore conclude that 2.5-s intervals from Cir X-1, as measured by modules 7 and 1-4, are more frequent than expected for randomly distributed events.

A random distribution of burst times fails to provide a good fit to the distribution of intervals between bursts, because there are many intervals near 2.5 s. Although this suggests a periodicity,

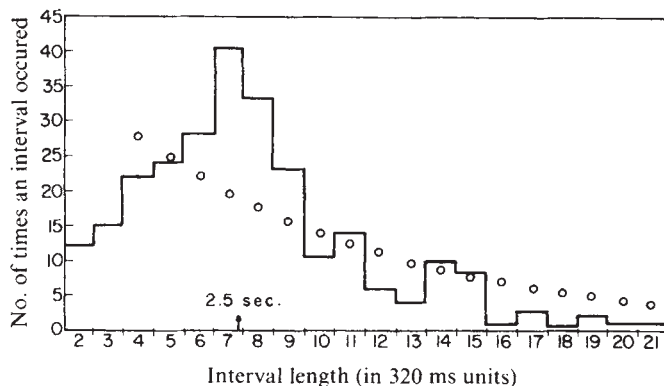


Fig. 3 The histogram shows the distribution of intervals between successive bursts seen in modules 1–4 and module 7. Circles represent the values that would be expected for a shot noise model.

and although highly periodic behaviour is seen on one scan, strict periodicity does not fit the observed intensity variations.

An alternative interpretation is that there is a characteristic delay following one burst before another can occur, and that in short data samples this sometimes gives the appearance of periodicity. This would mean that times of burst events are not completely independent, as in the shot noise picture.

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Structure of lithium–sodium β -alumina by powder neutron diffraction

THE first structural determination of a β -alumina compound containing two different alkali ions, lithium and sodium, in crystallographically distinct sites in the conducting plane is reported here. The lithium ions are displaced by about 1 Å from the mirror planes. Although such a displacement has been suspected it has not hitherto been confirmed.

Polycrystalline lithium–sodium β -alumina, with the two alkali species present in roughly equal proportions, has been shown to be a very good conductor of lithium ions¹. Although definitive

single crystal measurements have not been reported, the conductivity at room temperature, as measured by multi-frequency analysis of polycrystalline samples, is over 10^{-3} S cm⁻¹, the highest reported for any Li⁺ ion conductor. The ionic current is carried almost exclusively by lithium. The thermodynamic equilibrium is such that exchange of lithium for sodium above ~50–60% is difficult to achieve, and the ionic conductivity decreases if this is done. Such behaviour is in distinct contrast to the mixed alkali effect recently described² in sodium–potassium β -gallia, where, as in mixed alkali glasses, the ionic conductivity drops below that of either of the end members. This difference could arise if the structural distinction of the two alkali species, which we demonstrate in lithium–sodium β -alumina, is not a feature of most mixed alkali β -aluminas.

The structure of sodium β -alumina was determined in 1971 (ref. 3) and sodium atoms are found near the Beevers–Ross (BR) and mid-oxygen (MO) positions (Fig. 1). The structure of lithium β -alumina has not been reported, but the positive pressure coefficient of resistivity⁴ can be interpreted as indicating a displacement of lithium off the mirror plane at atmospheric pressure. Such a displacement would be expected from the known positions of the spinel block oxygen atoms and the effective ionic radii of lithium and oxygen⁵. When lithium is incorporated by ion exchange, as in the present work, it is expected to be present in the conducting plane. If incorporated during sintering and sample preparation, lithium will stabilise the β'' phase⁶ and is then presumably present in the spinel block, although this has not been demonstrated by structure analysis.

Table 1 Lithium–sodium β -alumina, PANDA diffractometer, $\lambda = 1.534$ Å (atom positions at 4.2 K)

Atom	Position	x/a	z/c	B (Å ²)	Occupation
O(1)	12k	0.15655(40)	0.04957(12)	0.14(5)	6
O(2)	12k	0.50220(48)	0.14682(11)	0.33(6)	6
O(3)	4f	2/3	0.05751(27)	0.40(14)	2
O(4)	4e	0	0.14233(35)	0.87(15)	2
O(5)	6h	0.29673(142)	1/4	0.70(36)	1
Al(1)	12k	0.83148(81)	0.10632(20)	0.76(7)	6
Al(2)	4f	1/3	0.02430(45)	0.55(15)	2
Al(3)	4f	1/3	0.17575(48)	0.83(17)	2
Al(4)	2a	0	0	0.46(22)	1
Li	6h	0.834(17)	(0.2086(34)	1.0	0.57(11)
Na	6h	0.7182(41)	1/4	1.0	0.44(5)

Cell parameters: $a = 5.5923(4)$; $c = 22.5576(6)$.

Data were collected on the PANDA diffractometer, Harwell ($\lambda = 1.534$ Å). The starting material was Monofrax sodium β -alumina containing 6.4% Na₂O (1.24 Na₂O : 11 Al₂O₃) which had been 60% exchanged with lithium in molten lithium nitrate. It was maintained at liquid helium temperature to minimise thermal motion effects which are difficult to distinguish from atomic displacements at higher temperatures in materials such as the β -aluminas, where several sites in the mirror plane may be partially occupied by the same atom. The data were refined using the profile refinement method described by Rietveld⁷.

The final atomic positions and thermal parameters are given in Table 1. The weighted profile agreement factor was 0.069. The accuracy obtainable using the powder technique does not allow a very precise determination of the occupations of the alkali ions, but the occupation numbers determined are close to those expected from the chemical composition (0.74 Li, 0.50 Na). The temperature factors of both atoms were constrained at 1.0 Å². Nor can we determine unequivocally by powder diffraction the structural basis of the observed non-stoichiometry in β -aluminas. The refinement in Table 1 assumes a

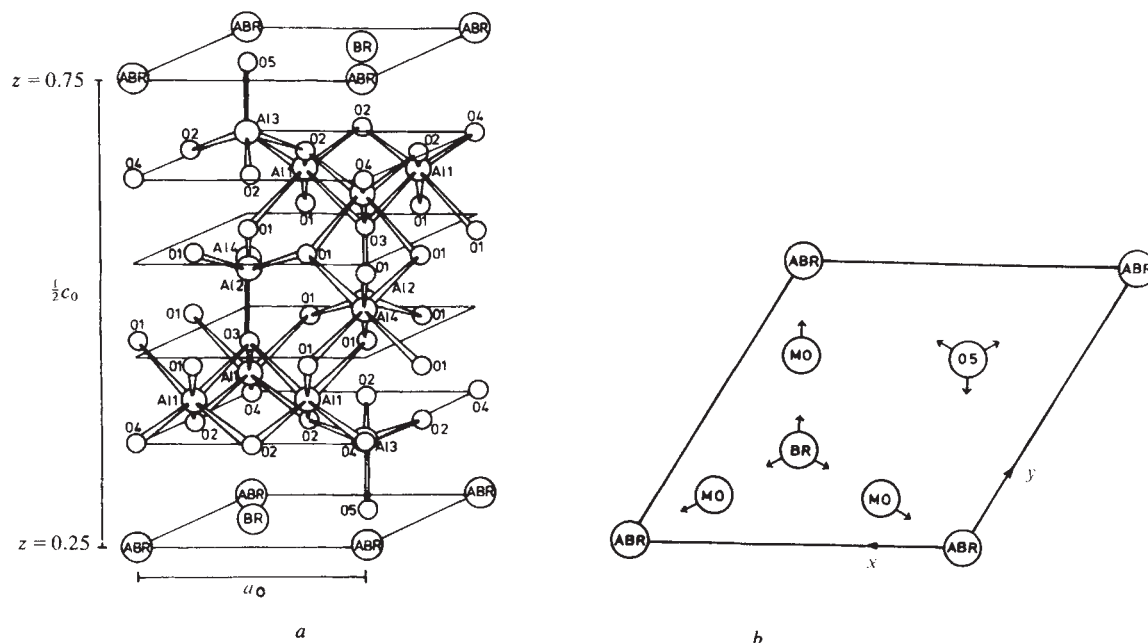


Fig. 1 The β -alumina structure. One half of the unit cell is shown in (a). A projection of the conducting plane is shown in (b) with the Beevers-Ross (BR), mid-oxygen (MO) and anti-Beevers-Ross (ABR) sites labelled. The directions of the small displacements often observed are indicated. For the sodium atom at the BR position in lithium-sodium β -alumina, there are three equivalent possible directions of displacement.

stoichiometric spinel block. However, incorporation of 0.24 interstitial aluminium displaced from Al(1) sites, and 0.12 oxygen atoms in the mirror plane close to the mid-oxygen sites in accord with the observed non-stoichiometry, causes a small increase in the lithium occupation to 0.65(12). The sodium occupation (0.47(5)) is virtually unchanged as are all other parameters. Both alkali occupations are then within one standard deviation of the expected values. This model of defect compensation, first suggested by Roth *et al.*⁸ for sodium β -alumina, and found for potassium β -alumina by Collin *et al.*⁹ seems, therefore, to be applicable in the present compound.

The relatively weak scattering powers of lithium and sodium for neutrons entail only a weak (1%) dependence of the *R*-factor on their presence. We believe, nonetheless, that the results obtained are meaningful, both because of the concordance of the occupations and positions with expectation, and because satisfactory refinements were not obtained with the alkali ions in alternative locations in the conducting plane.

The important structural conclusions about lithium-sodium β -alumina which may be drawn from the present work are then that the lithium and sodium are localised on crystallographically distinct sites and that lithium is displaced off the mirror plane. The sodium location, about 0.5 Å from the BR site, is very close to the dominant sodium location observed in sodium β -alumina³. Na-O(2) distances are 2.722(7) and 3.130(8) Å. The nearest Na-O(5) distance is 2.805(11) Å.

Lithium is displaced ~1 Å off the mirror plane and is located almost exactly over the MO site. Li-O(2) and Li-O(4) distances are 2.126(52) and 2.198(71) Å respectively. This displacement is necessary to reduce the Li-O distance to a value close to the sum of the ionic radii ($O^{2-} + Li(IV) = 1.99$ Å (ref. 5)).

Although we have shown the first structural distinction of two different alkali ions in the conducting plane of β -alumina, a structural determination on its own cannot explain the observed conductivity data¹. Long conduction paths are probably present for lithium motion without blocking by static sodium atoms; the role of the β -alumina framework and the sodium ions in promoting lithium ion mobility might be revealed by lattice statics or molecular dynamics calculation. The observed Li-O

distance, and, particularly, the minimum ABR-O(4) distance (2.43 Å), are all greater than the sum of the ionic radii. Therefore, we still need to know the optimum size of hole or slot to expedite rapid Li^+ motion, if indeed such a parameter can be defined without reference to a particular structure type.

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Interconnected fibre masses precipitated from solutions of polymers

IN studies to increase the mechanical strength of polymeric encapsulants for high voltage electronic devices we have formed fibre masses of unusual structure from isotactic polypropylene. The method for producing these three-dimensionally interconnected fibrous structures is described here and involves agitation of a solution of the polymer at sonic frequencies with simultaneous cooling. This process has since been observed for polyethylene and polybutene-1. Furthermore, the process has

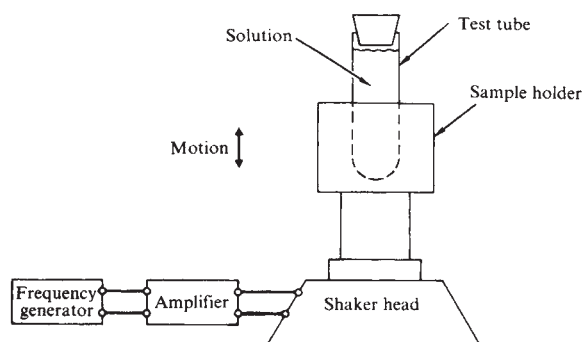


Fig. 1 Schematic diagram of experimental apparatus for low frequency agitation of polymer solutions.

been extended to other polymers, both crystalline and non-crystalline, through a process we call 'seeding'. Uniform reinforcement of encapsulating resins through the addition of high strength, short fibres, is not practical because of the complex shapes and numerous small interstices of the devices to be encapsulated. We therefore set out in 1973 to form fibres *in situ* by precipitation from low viscosity solutions which would readily impregnate a device of complex geometry. As a secondary objective, we hoped to polymerise the solvent to form a matrix around the fibres thereby producing a self-reinforced composite.

The fibrous crystal form of polymers was first reported in 1959 (ref. 1). This new crystal geometry, which consists of folded chain platelets regularly spaced on a central core of highly aligned polymer molecules, has been termed the 'shish kebab' structure. Pennings^{2,3} and Wikjord and St John Manley⁴ reported the formation of fibrous precipitates, almost entirely of the shish kebab structure, on the stirrer by stirring supercooled solutions of polyethylene and isotactic polystyrene respectively. Fibres have also been prepared by vigorous stirring of isotactic polypropylene, polyoxymethylene and polyethyleneoxide. Although the formation of high strength fibres by the creation of a sustained, essentially unidirectional, velocity gradient in a solution by stirring, high velocity jet, and other mechanical devices has become commonplace, there has been only one report of the use of high frequency agitation. Blackadder and Schleinitz⁵ found that irradiation of a dilute solution of polyethylene in *p*-xylene with ultrasonics from 85 to 190 kHz between 82 and 88 °C caused the formation of small fibrous crystals consisting entirely of the shish kebab structure.

Pennings⁶, in his study of the mechanics of fibrillar crystal growth, concluded that the creation of velocity gradients within the solution is the primary requirement. Extending this conclusion to the formation of fibre masses, we hypothesise that velocity gradients are generated in the solution by the sonic agitation. The hydrodynamic forces imposed on the coiled molecules by these velocity gradients cause them to extend completely or partially. Assuming that the thermodynamic conditions are suitable, the extended polymer chains and segments of chains have the opportunity to crystallise with each other in a substantially parallel or orientated manner. Segments of polymer coils which extend into the streaming solution, also undergo extension and orientation, thereby accelerating the growth process, and forming new branches of the original fibres. On cessation of the perturbing hydrodynamic forces, the segments of polymer chains extending from the partially orientated core of the fibre tend to crystallise in the usual folded chain configuration. They may account for the spherulitic and shish kebab-like structures which are often found mixed with the fibres.

In our initial experiments, we found that the stirring technique was an excellent way of establishing optimum conditions, such as

type of solvent, polymer concentration and temperature, for fibre deposition. Stirring, however, causes fibres to form on and extend from only the exterior surface of the rotating member and therefore was not suitable for forming fibres in and around complex shapes. Ultrasonic formation of fibres was ineffective; the yield of fibres was sparse and the fibres were discrete and very short. We then decided to try the effect of agitation at sonic frequencies on relatively concentrated polymer solutions. The apparatus used for these experiments is shown in Fig. 1. We placed a test tube of hot polypropylene solution in the sample holder and subjected it to low frequency agitation as it cooled. At a few degrees below the dissolution temperature dense fibre masses began to form in the solution. These masses grew until they filled the entire fluid volume of the container. The fibre masses were usually so densely interconnected that they had good mechanical integrity and retained the entire volume of the solvent.

Isotactic polypropylene proved to be most amenable to the formation of fibrous masses. Concentrations of 0.7–20% polypropylene were dissolved in *p*-xylene at 132 °C and allowed to cool while the frequency of vibration was scanned in the 80–1,000 Hz range. Dense fibrous mats were formed. In other experiments, fibre masses were formed from concentrations of 5% and 10% isotactic polypropylene in styrene monomer inhibited with 0.5% hydroquinone. The styrene-impregnated fibre plugs were then placed in an oven at 70 °C to polymerise to monomer into a plastic matrix of polystyrene to form a self-reinforced composite.

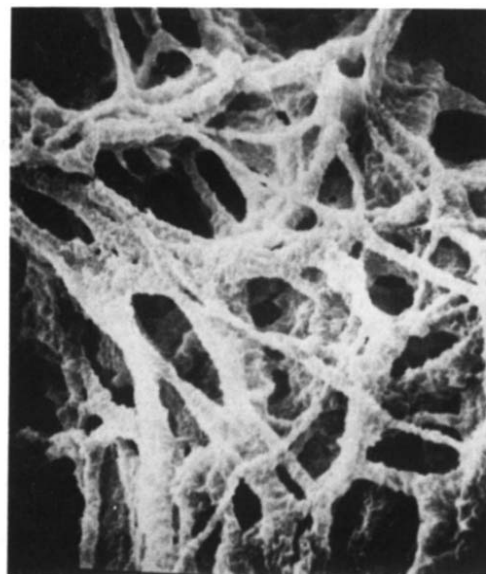


Fig. 2 Polypropylene fibre mass showing branching and interconnection. Scanning electron micrograph ($\times 4,800$).

Interconnected fibrous masses have been formed from polyethylene, isotactic polybutene-1 and isotactic poly-4-methylpentene-1. Polypropylene has also been used to induce other linear polymers, including Nylon 6, acrylonitrile butadiene styrene terpolymer, polystyrene, polyvinylidene fluoride, and tetrafluoroethylene hexafluoropropylene vinylidene fluoride terpolymer, to co-deposit in fibrous masses. This is the technique which we have called seeding. It is possible that the phenomenon of fibre mass formation under sonic agitation is general for isotactic crystalline polymers provided the optimum solvent system, concentration range and precipitation temperature is selected. Furthermore, the technique can be extended by seeding to many other linear polymers.

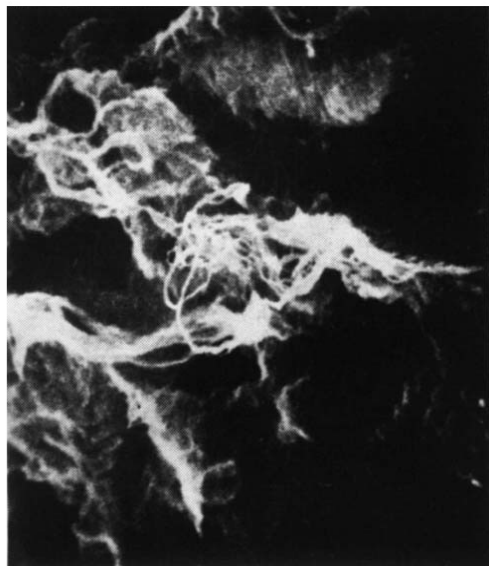


Fig. 3 Fibrillar structure of fibres from fibre mass scanning electron micrograph ($\times 11,000$).

The morphology of the fibrous masses depends on many factors including concentration, rate of cooling, solvent type and the frequency and effectiveness of the agitation. In general, the most uniform, highly orientated, fibrous and highest strength fibre masses are produced from fairly low concentrations at very slow rates of cooling during precipitation. The precise mechanism of fibre mass formation by sonic agitation is not understood. We do not know whether the hydrodynamic shearing forces necessary for initial fibre formation are produced by cavitation, interaction of the solution with the walls of the container or some combination of perturbations. The following general observations may be helpful. Isotactic polypropylene very readily forms fibre masses from several solvents such as *p*-xylene, styrene, toluene, and mixtures of styrene, acrylic acid and methylmethacrylate. Lower viscosity solutions form fibres more readily than viscous, concentrated solutions. Efficient coupling between the container of the polymer solution and the shaker is important for the formation of uniform fibre masses. Frequency of agitation in the sonic range does not seem to be important except that those frequencies which are resonant with the container of polymer solution will cause the most efficient agitation and result in the most uniform fibrous masses. Also, a small air-filled space between the cap of the container and the surface of the solution seems to be necessary. Fibre plugs will not form in a full test tube which is sealed with an impervious rubber stopper but will form when the rubber stopper is replaced with a cork.

The structure of a well formed fibrous mass is shown in Fig. 2. If the solution is cooled too rapidly, or is too concentrated, the fibres will be co-deposited with spherulites and flocculent material. The fibres vary from 300 Å to about 0.3 mm in diameter and are variously branched or bifurcated to form an interconnected, three-dimensional network. Examination of the fibres at high magnification reveals that they seem to be made up of essentially parallel arrays of fibrils (Fig. 3).

We and our co-workers have subsequently formed fibre masses by a variation of the sonic agitation technique which permits more precise control of the experimental conditions. Fibres from these experiments have the shish kebab morphology and greatly improved strength over the bulk polymers. This work will be reported elsewhere.

This study was supported as a Hughes Aircraft Company Independent Research and Development Program and has

resulted in the filing of several patent applications to cover fibre masses and the 'seeding' technique. We thank C. G. Walance and G. R. Hokenstad for their support and R. E. Kelchner for his technical advice.

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Sea-level changes during the late Pleistocene and Holocene in the Strait of Malacca

CURRENT concepts of late Pleistocene sea level history, generally referred to the ^{14}C time scale, differ considerably¹. Some authors^{2,3} assume that the sea level at about 30,000 BP was comparable with that of the present and others^{4,5} assume a considerably lower sea level at that time. We have now obtained ^{14}C dates from *in situ* roots and peat which indicate that the sea level was lowered eustatically to at least 40–60 m below the present level between 36,000 and 10,000 BP. The sea level rose from –13 m to about +5 m from 8,000 to 4,000 BP and then approached its present level.

A combined coastal and offshore survey in the Strait of Malacca between Port Dickson and Singapore yielded 33 reliable ^{14}C ages obtained from autochthonous organic material such as peat and roots (Table 1). In the coastal region, samples were collected by hand augers which could penetrate up to 18 m of unconsolidated sediments. In the offshore area a shipboard-operated vibrocorer was used which recovered cores up to 3 m long. Samples were obtained between 75 m below and 6 m above mean sea level.

The geological settings of the dated layers cannot all be used as direct indicators of sea level but can serve as indirect indicators. In the offshore zone, the substratum of the peats is mostly unknown, because the vibrocorer did not penetrate the peat layers. Some of these peats may have been formed in association with freshwater lakes, as indicated by the presence of a diatom ooze. The upper parts of the peat and their possible sedimentary cover were eroded during the Holocene transgression or by sub-Recent tidal currents. Today the peats, which have a fresh and unweathered appearance, are mostly overlain by sandy marine sediments. Data obtained from this type of peat indicate only that the sea was below the sampled level during the time of peat formation. The same conclusion can be drawn from *in situ* roots in terrestrial sediments.

The Holocene basal peats of the coastal sedimentary sequences were formed on top of terrestrial deposits usually with no erosional hiatus between them and the overlying marine sediments. In this case it can be assumed that the sea invaded the area shortly after the phase of peat formation.

In wide areas of the coastal lowlands, peat occurs as an organic cover on the top of clastic deposits. This overlap of a semi-terrestrial facies over brackish and marine facies indicates the beginning of regressive tendencies in the coastal development. Mangroves are direct indicators of the tidal zone between mean neaps high and mean springs high (at present ~1.3 m).

Depth distribution of ^{14}C ages of autochthonous organic material of the sampled core sections (Figs 1, 2) shows that

Table 1 ^{14}C dates from *in situ* roots and peat

^{14}C age BP	Lab. no.	$\delta^{13}\text{C}$	Geographical coordinates		Relation to mean sea level (m)	Dated material
1,055±85	Hv 7598	-27.6	2°31' N	101°46' E	-0.65 to -0.75	Mangrove wood on top of terrestrial deposits
1,145±80	Hv 7603	-27.3	2°25' N	101°58' E	-0.60 to -0.63	Mangrove wood on top of marine sediments
2,130±155	Hv 7588	-27.7	1°46' N	103° 1' E	+1.80 to +2.00	Basis of peat layer on top of brackish sediments
4,040±100	Hv 7583	-29.9	1°37' N	103°25' E	+5.85 to +5.90	Mangrove and peat above terrestrial deposits
4,135±90	Hv 7589	-31.8	2° 1' N	102°47' E	+4.95 to +5.00	Peat on top of brackish deposits
4,275±70	Hv 7587	-29.5	1°48' N	103°15' E	+4.70 to +4.95	Peat on top of brackish deposits
4,335±105	Hv 7586	-31.0	1°43' N	103°18' E	+2.75 to +2.80	Peat to fresh water deposit on top of brackish sediments
4,980±75	Hv 7580	-29.5	1°29' N	103°23' E	+3.70 to +3.80	Basis of peat layer on top of brackish deposits
5,975±75	Hv 7594	-28.4	2°19' N	102° 5' E	+1.10 to +1.25	Mangrove wood at the base of coastal barrier sand
6,260±75	Hv 7582	-29.2	1°32' N	103°28' E	+0.63 to +0.67	Peat to mangrove above terrestrial deposits
6,610±70	Hv 7581	-27.8	1°32' N	103°28' E	+0.80 to +0.95	Mangrove wood
6,945±120	Hv 7584	-27.2	1°37' N	103°25' E	-0.35 to -0.50	Peat on top of terrestrial deposits
6,985±180	Hv 7601	-28.8	2°22' N	102° 0' E	-3.70 to -3.80	Mangrove wood
7,015±80	Hv 7592	-29.3	2°10' N	102°36' E	-2.55 to -2.90	Peat on top of terrestrial deposits
7,175±70	Hv 7595	-27.9	2°25' N	101°56' E	-5.00 to -5.10	Wood from a thick sequence of mangrove deposits
7,340±100	Hv 7591	-27.5	1°59' N	102° 40' E	-5.00 to -5.10	Peat on top of terrestrial deposits
7,440±175	Hv 7596	-30.2	2°25' N	101°56' E	-5.80 to -5.85	Wood from a thick sequence of mangrove deposits
7,560±135	Hv 7597	-29.0	2°25' N	101°56' E	-7.50 to -7.55	Wood from a thick sequence of mangrove deposits
7,985±220	Hv 7593	-29.5	2°12' N	102°18' E	-12.65 to -12.85	Wood from a peat on top of terrestrial deposits
8,490±85	Hv 7650	—	1°51' N	102° 9' E	-21.10 to -21.20	Peat on top of marine muds
9,265±105	Hv 7644	-28.7	1°13' N	103°12' E	-33.41 to -33.42	Woody peat overlain by marine mud
9,840±330	Hv 7669	-26.5	2°20' N	101°44' E	-53.40 to -53.70	Peat overlain by marine sand
10,590±190	Hv 7646	-14.2	1°16' N	103°11' E	-33.65 to -33.75	Clayey peat with <i>in situ</i> roots on top of terrestrial silty clay
12,440±705	Hv 7643	-23.7	1°14' N	103°14' E	-38.45 to -38.65	<i>In situ</i> roots in weathered quartzite
17,170±110	Hv 7662	-30.4	2°39' N	101°28' E	-56.95 to -57.09	Woody peat overlain by marine mud
23,800±2,550	Hv 7663	-21.3	2°36' N	101°31' E	-54.00 to -54.10	<i>In situ</i> roots within terrestrial clay
1,930						
26,330±3,410	Hv 7653	-31.7	1°59' N	102°17' E	-35.30 to -35.60	Leaves and wood from a fresh water diatom ooze
2,380						
27,400±1,000	Hv 7665	-29.8	2°34' N	101°30' E	-65.60 to -66.00	<i>In situ</i> mangrove (?) roots
890						
32,800±2,170	Hv 7648	-30.2	1°30' N	103° 7' E	-34.70 to -35.60	Leaves from freshwater (?) silty clay
1,700						
33,670±2,100	Hv 7664	—	2°35' N	101°30' E	-67.20 to -67.25	Peat overlain by marine sand
1,660						
37,700±1,810	Hv 7640	-30.3	0°44' N	103°18' E	-16.50 to -16.52	Sand impregnated with humate
1,480						
41,650±3,850	Hv 7641	-25.5	0°43' N	103°20' E	-17.60 to -17.70	Peat overlain by marine sand
2,600						
>44,200	Hv 7652	-27.9	2° 0' N	102°20' E	-36.13 to -36.15	Peat overlain by marine mud

between approximately 40,000 and 10,000 BP continental conditions prevailed in most parts of the southern Malacca Strait. Between 40,000 and 38,000 BP peat was formed at 17 m below present sea level, and around 34,000 BP at -67 m. Leaves and wood found at -36 m in freshwater diatom oozes gave ^{14}C ages of 32,800 and 26,300 BP. *In situ* roots, presumably from a former mangrove swamp at -65 m and with a ^{14}C age of 27,400 BP, give the only direct time-depth position of a Pleistocene sea level. *In situ* roots from terrestrial sediments and peat grew at -55 m between 23,800 and 17,200 BP. Three additional peat dates from the Strait of Malacca^{6,7} and the southern Gulf of Siam⁸ supplement these dates. From 8,000 to 6,000 BP the sea level rose from -12.8 m to +1.2 m relative to the present level.

Sea level indicators for the time interval between 5,000 and 4,000 BP have been found at +2.5 m to +5.8 m, which is the highest dated level. The subsequent lowering of the sea level to its present value is indicated by a peat sample at +2.0 m with a ^{14}C age of 2,000 BP and two mangrove samples at -0.8 m, dated at 1,000 BP. This tendency is consistent with the results of Tjia *et al.*⁹. However, our data do not show whether the lowering of the sea level took place steadily or through an oscillatory process.

The reliability of conclusions concerning sea level history is limited by the errors involved in ^{14}C dating, and in correlating

the sample sites with the corresponding sea levels. For the dating of our offshore peat samples, only contamination by younger roots need be taken into account. Because of the fresh appearance of the peats and their stratigraphical position below the marked marine erosional horizon, such roots were not expected and they were not found. These roots would also have to be older than 10,000 yr because of the Holocene transgression, and in our experience contamination by finely dispersed macroscopically invisible roots is generally lower than 3%. With the most unfavourable of these assumptions, samples with actual ages of 100,000, 40,000, 30,000, and 20,000 yr would correspond to ^{14}C ages of 38,000, 33,600, 27,700 and 19,500 yr, respectively. In other words, the ^{14}C time scale seems to be increasingly compressed towards the earlier end of the scale.

The amount of compaction which may have changed the depths of the sea level indicators is unknown for the offshore area. For the coastal area it can be estimated roughly to be up to 5.5 m. This is the height difference between the highest sea level indicator in the sedimentary sequences and the corresponding level of a marine abrasional platform on weathered granite.

The curve for Holocene sea level changes in the Strait of Malacca compares well with those areas which are considered to

Table 1 The maximum fraction f of an area covered by randomly placed particles

Method: coarse mesh (except as indicated)								
Periodic boundary					Rigid boundary			
Current work					Current work		Akeda & Hori ⁷	
Size of area ($N \times M$)	Number of trials	Average lower bound	Average upper bound	Standard deviation of upper bound*	Average lower bound	Standard deviation	Mean	Standard deviation
20 × 30	40	0.5653	0.5677	0.0089	0.5509	0.0140	0.5473	0.0096
31 × 31	40	0.5633	0.5652	0.0089	0.5525	0.0090		
31.5 × 31.5	20		0.5538†	0.0080†				
40 × 40	40	0.5629	0.5649	0.0053	0.5537	0.0061	0.5534	0.0043
60 × 60	40						0.5567	0.0040
100 × 100	40						0.5592	0.0023
200 × 200	10						0.5610	0.0012

Circular particles

Method, fine mesh; periodic boundary conditions; size of area, $1,024 \times 1,024$ fine squares; particle diameter, 27.5 fine squares; 10 trials.

* Standard deviation of lower bound was virtually the same.

† Fine mesh; not an upper bound, but a mean.

Mean 0.5027. Standard deviation 0.0033.

in cell membranes. When the membrane bilayer is split into two leaflets by freeze-fracture method⁴ and an area of the split membrane is visualised by electron microscopy, then the embedded membrane particles (believed to be predominantly proteinaceous) are seen as circular shapes. In natural membranes the unaggregated particles seem to be random in position, and the areal density of the particles may be high. It seems that nature has provided us with a two-dimensional example of (nearly) maximum random parking. Hence we have estimated the maximum fractional area covered by circular disks on a two-dimensional membrane, using a computer simulation with periodic boundary conditions. The correspondence with particles in a biological membrane has implications for membrane biogenesis.

The two-dimensional random sequential placing ('car parking') problem may be described as follows. Cars, each of the same size, are dropped from the air one at a time into the empty space of a parking lot. The place to park each car is chosen so that there is a (uniform) probability of parking on the remaining empty space. When no more cars can be parked (without damaging one another) then the parking lot is 'saturated', and the fractional area f of the lot which is occupied by cars is the quantity of interest. Palásti⁵ conjectured from the one-dimensional case that in two dimensions $f \sim 0.5589$. Previous simulations of the two-dimensional case^{1,5-7} have taken the cars to be square, with sides always parallel to those of a square parking lot of finite extent ('rigid boundary'). Here, Monte Carlo analogues are used to find f for circles and for squares, with periodic boundary conditions being novel in the present context. The choice of computing technique is most important, as it is affected by the expense of computation time.

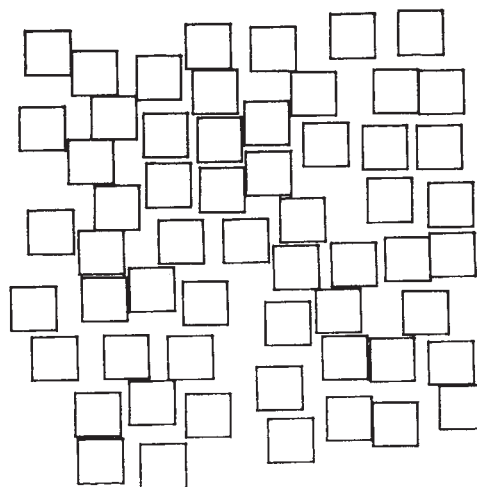
We used two basic methods for placing particles by computer: (1) the coarse-mesh method for placing particles only; and (2) the fine-mesh method for square and disk particles. They will be described in detail elsewhere³.

Method (1) is an improvement of that of Akeda and Hori^{6,7}. Some results are shown in Table 1. The principle of the fine-mesh method is that the parking lot is covered by a fine mesh of squares which are much smaller than a particle: a particle (disk shaped) is centred on a square, and the particle is then represented by the minimum set of fine squares which will completely cover the area of the particle. To obtain reasonable accuracy (to three significant figures in placement) the square 'parking lot' was divided into a mesh of 1024×1024 fine squares: the

availability status (whether or not a fine square was available for a particle centre) was stored as a bit in a single megabit array. Square particles (used for checking the fine-mesh method against the large-mesh method) were $(65/2) \times (65/2)$ fine squares in size; circular particles were of diameter $55/2$ fine squares.

The essence of the fine-mesh method is that, by careful counting, the problem of making many trials to avoid overlaps is eliminated, which is unavoidable in the large-mesh method. As the centre location for each particle is randomly selected directly from those fine meshes available, then the neighbouring region thereby excluded to another particle is also determined and recorded in the megabit array: this region will be a square of 65×65 or a circle of diameter 55 fine meshes. The areal error due to representing the smooth outline of a circle by an outline made from many small squares is much less than 1.5%.

To identify directly the next vacant fine squares on which a particle centre might be randomly placed, two additional $1,024$ long integer arrays are used (that is, two integers for each row of the $1,024 \times 1,024$ array) for the for book-keeping purposes.

**Fig. 1** Sample of large mesh method for randomly sequentially placing square particles into a square region.

To produce plots such as Fig. 2, an integer array is used to record the coordinates of the particle centres. To reduce the particle placement time, the shape of one-quarter of a particle is stored in an integer array (further details required to make the method feasible are given in ref. 3).

A great advantage of the method is that the computer time required to find a location for each particle is the same for each placement (parking). Particles are placed until there is no space available for a particle centre. While the time required to place a particle is quite long (48 ms to place each $(65/2) \times (65/2)$ square particle, including all book-keeping, on IBM 370/168), it remains independent of the number of particles placed. (The time and memory requirements of the program increase drastically with the accuracy of the placement desired. To add one

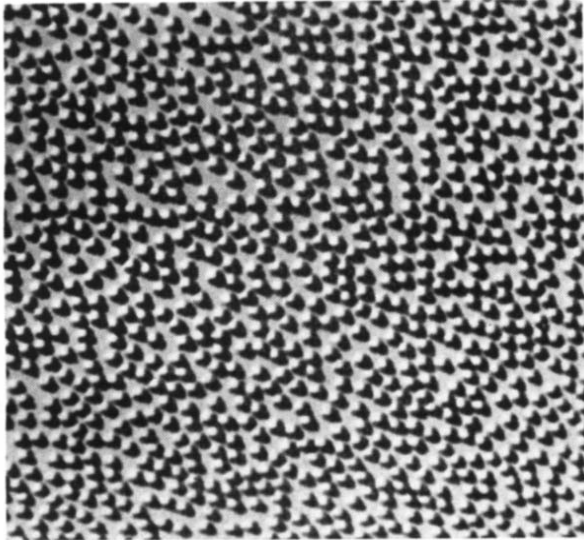


Fig. 2 Sample of fine mesh method for randomly sequentially placing circular particles (disks) into a square region. (The area shown is covered by a square mesh—not shown—of $1,024 \times 1,024$. Also, the disks have been synthetically shadowed so as to simulate the appearance of freeze-fractured particles, which usually seem to be hemispherical.)

significant figure of accuracy in the two dimensions would increase both the time and memory requirements of the computer program 100-fold.)

The new result for the fractional area covered by maximally packed disks is 0.5027 ± 0.0020 . We are not aware of any theoretical (or previous experimental) estimate (and simply note that this result is not inconsistent with the difference in shape between circles and squares, and that the squares were all orientated with sides parallel). For the maximum fractional area f covered by non-overlapping orientated square particles on a surface, in addition to Palásti's⁵ conjecture of 0.5589 and Akeda and Hori's⁷ extrapolation of a simulation with rigid boundary conditions of 0.5626 ± 0.0006 , we now have simulations with more realistic periodic boundary conditions which do not require extrapolation. These new results are for the coarse-mesh method $f = 0.5629 \pm 0.0016$ (lower bound) and $f = 0.5649 \pm 0.0016$ (upper bound) (errors are 95% confidence limits about the estimated mean), and for the fine-mesh method $f = 0.5538 \pm 0.0035$. (The nominally low error quoted⁶ is statistical, due to taking many samples, and will not affect the methodological problem associated with rigid boundaries in their simulation.) These new results bridge Palásti's conjecture⁵. Hence, in contradiction to other simulations⁷ these new results together show that simulations give no reason to reject her⁵ conjecture for orientated squares.

The areal density of particles in the human erythrocyte membrane² is substantially close to that of the maximum placing

of disks. As during membrane biogenesis^{8,9} proteins may be inserted into the membrane in a sequential process, it seems that to avoid the time and energy costs of many insertion attempts, the membrane must permit the protein to move laterally after insertion, that is, there must be at least short-range membrane fluidity⁹.

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The greening of polar bears in zoos

POLAR BEARS (*Thalarctos maritimus*) normally have creamy-white fur, presumably an adaptation for camouflage in a snowy environment. However, during the summer of 1978, the fur on the back and sides of three adults in the San Diego Zoo turned green, though the animals remained otherwise healthy. (Of these bears, one female was born in the zoo in Calgary, Alberta, Canada, in 1966 and was transferred to the San Diego Zoo in 1969; a second female was born in the wild, having been caught at Spitzbergen in 1951; the third, a male, was born in the San Diego Zoo in 1970.) This phenomenon, though less marked, has been noted in several previous summers, both here and in zoos elsewhere, for example, in Cologne, Germany (C. Hill, personal communication). The coloration was particularly evident on the flanks, on the outer fur of the legs and in a band across the rump: fur on the head and belly and inner sides of the legs was white. We first supposed that the colour was due to green algae such as *Chlorella* or *Scenedesmus* on the surfaces of hairs, growth of such algae being promoted by the presence of nitrogenous wastes in the waters of the bears' pool. (The pool in the exhibit area, which contains 12,500 gallons of tap water, is drained and cleaned twice weekly.) However, microscopic examination of samples of hair taken from the three San Diego bears and from a similarly green polar bear in the zoo at Fresno, California, revealed that this was not so. The outer surfaces of the hairs appeared clean and smooth, except for the normal squamation. The coloration was clearly attributable to the presence of algae inside the hairs, specifically in the hollow medullae of many of the wider (50–200 μm), stiffer guard hairs of the outer coat. (The thinner (<20 μm) and more undulant hairs of the under coat, which were not hollow, were colourless.) Some of the lumina were apparently filled with air, but many of these hollow spaces were partly occupied by masses of small greenish cells, which we describe here.

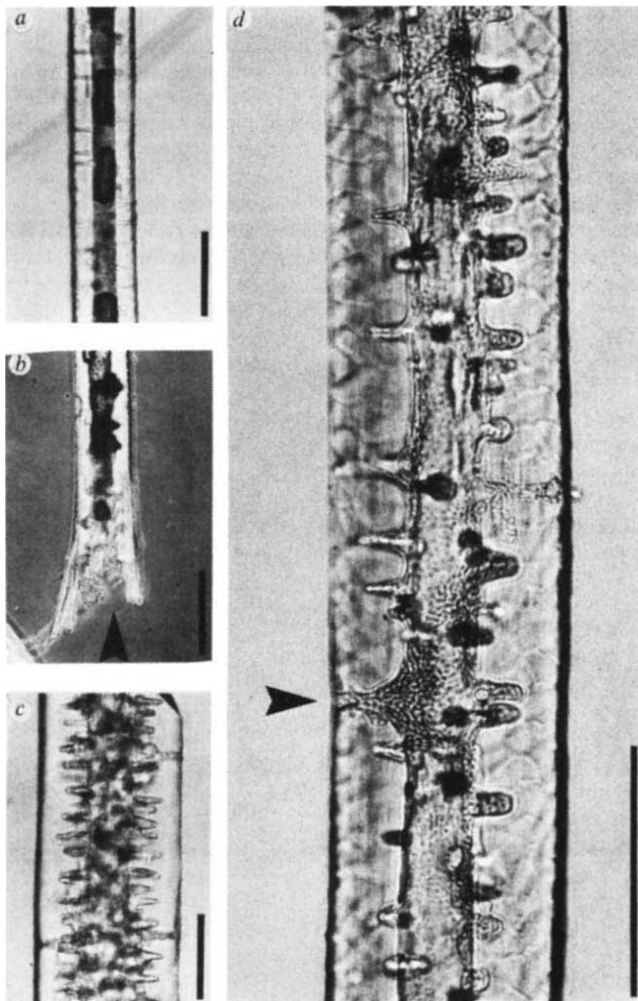


Fig. 1 *a*, Portion of green polar-bear hair, showing medulla containing air cavities and sections filled with algal cells. Note lateral ducts. *b*, Broken end of green polar-bear hair, showing medulla containing algal cells. *c*, Portion of green polar-bear hair, showing medulla with numerous lateral ducts. *d*, Portion of green polar-bear hair, showing medulla with lateral ducts (some communicating with outer surface: arrow) containing algal cells. Phase-contrast; scale bar, 100 μm .

The interior surfaces of the lumina of the narrower guard hairs were smooth. Many of those of intermediate width had pits and channels, more or less perpendicular to the hair axis, extending part of the way to the surface. Some were expanded into chambers parallel with the hair axis, especially near the broken distal end. Those of the stoutest hairs had very many such transverse channels (5–10 μm wide), some extending fully to the outer surface. These features, visible by light microscopy (Fig. 1*a, b*), could be clearly seen in stereoscan electron micrographs of sectioned hairs (Fig. 1*c, d*), which also showed pits in the hair surfaces where some of the side channels opened to the outside (Fig. 2*a, b*).

We have been unable to find any reference to such lateral ducts in the hairs of any mammal¹. Hairs of comparable size from the head, shoulders and flanks of two wild polar bears from Spitzbergen, killed at Isfjord in May 1912 and at Svalbard in July 1967 were white, as were those of three bears obtained this year at L. Churchill, Manitoba: they had similarly hollow medullae but lacked any signs of lateral channels or of algae. (Hairs from four other marine mammals, a sea-otter (*Enhydra*), a northern fur seal (*Callorhinus*), a California sea-lion (*Zalophus*) and a harbour seal (*Phoca*), appeared solid: they too were alga-free.) It is possible that the transverse ducts were formed by keratolytic bacteria, which initially entered the lumina through

broken distal ends. However, in the sections of green bear hairs examined by electron microscopy we saw little evidence of bacterial degradation of the keratin (Fig. 2*c, d*).

The fact that some of these lumina were in connection with the external air or water could explain how the algal cells could have entered the hairs in the first place, and how exchange of O_2 and CO_2 and uptake of water and mineral salts would be facilitated and could permit growth of the algae if suitably illuminated. Indeed, such a habitat has certain advantages, being warm and protected from most kinds of potential predators.

The algal cells from the green zoo bears were spherical, 2–4 μm in diameter. They were clearly prokaryotic; no plastids or nuclei could be distinguished, either by phase-contrast microscopy or in thin sections examined under the electron microscope. They divided by equatorial constriction; no other mode of reproduction was seen. In size and form they resembled *Aphanocapsa montana* Cramer, a blue-green alga in the order Chroococcales, though they appeared more green than blue-green.

Pure cultures were obtained by grinding a washed sample of the green hairs with sterile mineral nutrient medium in a Potter-type homogeniser and streaking a drop of the supernatant algal cell suspension on a 1% agar medium containing mineral nutrient salts². The cells were constantly illuminated at ~2,000 lux from cool-white fluorescent lamps at 27°C. After a week, individual algal colonies were visible under low magnification. Virtually all colonies were of the same size and form, confirming what had already been indicated by microscopic examination of the hairs, that is, that their algal cells were almost all of one type. (Among hundreds of thousands of colonies, only a dozen were of an evidently different kind: a filamentous blue-green alga, similar to *Phormidium*, with cells <2 μm wide.) Apparently clean colonies were selected, transferred to liquid Maertens' medium and illuminated in the same way. When tested on medium supplemented with 0.1% yeast extract, although most were contaminated with bacteria, a few were axenic. Pure cultures were subcultured in Maertens' medium in flasks on a shaker; they grew well, and were comparable in colour, cell size (2.5–4.5 μm), form and fine structure with those observed in the bear hairs (Fig. 3*a*). They required no organic factors for growth. In older cultures, possibly depleted

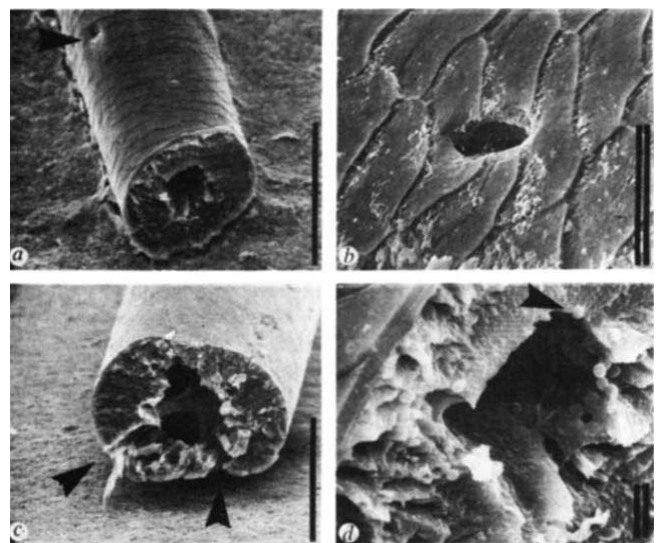


Fig. 2 *a*, Cut end of green polar-bear hair, showing hollow medulla and lateral pore (arrow) among imbricate scales. Stereoscan electron micrograph (SEM); scale bar, 100 μm . *b*, Pore among imbricate scales on surface of polar-bear hair. SEM; scale bar, 100 μm . *c*, Cut end of green polar-bear hair, showing hollow medulla with lateral ducts opening to surface (arrows). SEM; scale bar, 10 μm . *d*, Cut end of green polar-bear hair, showing algal cells (arrow) in lumen. SEM; scale bar, 10 μm .

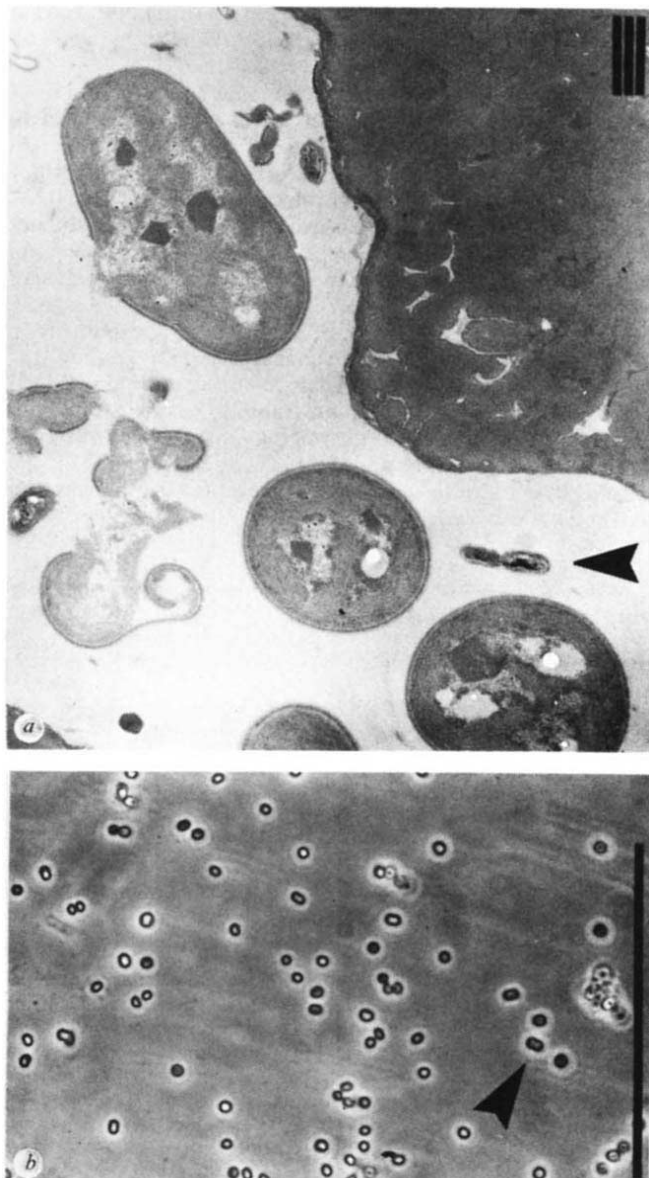


Fig. 3 *a*, Thin section of green polar-bear hair, showing cells of cyanophytes and bacteria (arrow) in medullary space. Transmission electron micrograph (TEM); scale bar, 1 μ m. *b*, Cells of *Aphanocapsa montana* (?), showing division stages (arrow), in clonal culture isolated from green polar-bear hair (San Diego Zoo). Phase-contrast; scale bar, 100 μ m.

of a fixed source of nitrogen, the cells became enveloped in colourless mucilaginous sheaths (Fig. 3*b*) which often developed asymmetrically. Growth was most rapid at 33 °C; above 36 °C the algae did not grow. When tested in a range of salinities they behaved like many freshwater species; at salinities below one-third sea water they grew well, but at higher concentrations growth was inhibited. Although we know of no record of keratin digestion by any alga, we tested for this by incubating sections of white polar-bear hairs with a pure culture of this alga in a mineral medium constantly shaken with air at 27 °C in white light. After 12 weeks, although cells grew in the lumina, we could see no indication of erosion of the hairs.

The cultured algal cells were usually bright green. When extracted with non-polar solvents they were found to contain chlorophyll *a* (but no chlorophyll *b*) and various carotenoids. When grown in white light, the cells produced no detectable phycobilin pigment; but when grown in red light they developed a distinctly bluish tinge and formed appreciable amounts of phycocyanin (tested by *in vitro* absorption and fluorescence spectra). We therefore concluded that the alga is a cyanophyte.

Similar cultures were isolated in the same way from hairs of the green polar bear at Fresno, mentioned above. Apparently the alga commonly infests the hairs of polar bears in zoos. It is a freshwater species, the cells of which probably originate from the pond water in the bears' enclosures and enter the medullary lumina of the guard hairs through broken distal ends or through the patent lateral ducts.

Few other chordates are known to harbour algae on their surfaces. Certain colonial ascidians in the family Didemnidae are associated with prochlorophytes³. Freshwater turtles may bear branched filamentous chlorophytes in the genus *Basycladia*⁴. Unidentified green algae may colour the pelage of monk seals (*Monachus*)⁵, while brown or red algae (*Ectocarpus*, *Erythrocladia*) have occasionally been found growing on the hairs of fur seals⁶. On two-toed and three-toed sloths (*Bradypus* and *Choleopus*) grooves on the outer surfaces of their guard hairs bear algae of two kinds; filamentous chlorophytes assigned to the genus *Trichophilus* (Chaetophorales) and purple cyanophytes which have been called *Cyanoderma* (Chamaesiphonales)⁷. Several genera of pennate diatoms grow on the skin of various kinds of whales, including the 'sulphur-bottomed' whale⁹. These algae are all quite unlike the species found in hairs of captive polar bears, described here. The African prosimian primate *Galagoides* has been reported to have green algae associated with its fur⁸, but this could not be confirmed.

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Receptor for IgG(Fc) and human β_2 -microglobulin on *S. mansoni* schistosomula

HOST antigen uptake by schistosomes is generally considered from circumstantial evidence to be a potent mechanism by which schistosomes can evade immune response and has therefore stimulated several investigations on the chemical nature of the molecules as well as their possible mode of incorporation¹. These studies have indicated that the parasite can acquire host material in the form of glycolipids of erythrocyte origin² and gene products of the murine major histocompatibility complex³. As an alternative to host antigen incorporation, the possibility of immunological blockade has been suggested. Host immunoglobulins have been demonstrated to be associated with the tegumental surfaces of *Schistosoma mansoni* from mice and baboons, and recent experiments^{4,5} have shown the presence of

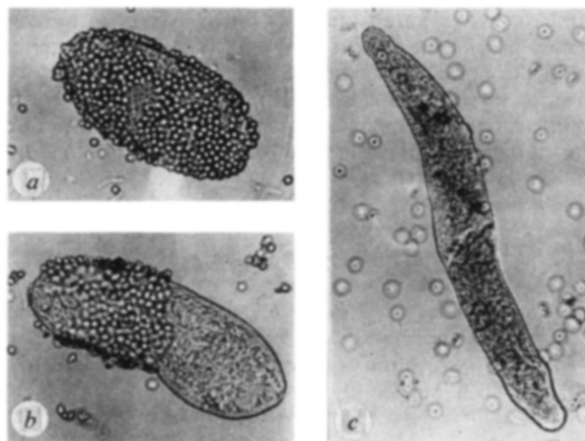


Fig. 1 Photomicrographs ($\times 100$) of characteristic rosette formation (EA) with sensitised sheep RBC on the surface of different life stages of schistosomula. Schistosomula collected *in vitro* after skin penetration show complete rosetting EA (a). Schistosomula obtained after mechanical disruption of the cercarial tail show EA rosettes capped (b) at the anterior part of the parasite. No rosette formation is observed when 5-d-old schistosomula collected by lung recovery are used (c).

heterospecific antibody on the tegumental surface of *S. mansoni* adult worms. The ability of adsorbed antibodies to bind their respective antigens indicates that some of these immunoglobulins are orientated so as to present at least one free Fab region of the antibody molecule. Although this could merely represent a nonspecific adsorption, it could also represent the specific binding of the immunoglobulin on the parasite's surface. Using a rosette test we have now demonstrated the presence of receptors for both IgG(Fc) and human β_2 -microglobulin.

Sheep erythrocytes (RBC) were sensitised with mouse anti-sheep erythrocyte antibody (EA) to form rosettes, after which the cells were washed and placed in contact with schistosomula obtained *in vitro* after skin penetration according to Clegg and Smithers⁶. In other experiments schistosomula were incubated for 30 min with mouse anti-sheep RBC antibody, washed and placed in contact with normal sheep erythrocytes. In these two experimental conditions, rosette formation was observed around schistosomula which seemed to be almost totally coated with erythrocytes (Fig. 1a). When cercariae collected before skin penetration or 5-d-old schistosomula (Fig. 1c) collected by lung recovery in infected mice were used, no rosette formation was observed (Table 1). Schistosomula, obtained without skin penetration, after mechanical disruption of the cercarial tail⁷,

Table 1 Rosette formation at different life stages of *Schistosoma*

	Sensitised sheep RBC	Sheep RBC
Cercariae	—	—
3-h skin preparation schistosomula	+++	—
Artificially transformed schistosomula	++	—
5-day lung schistosomula	—	—
Adult worms	—	—

Sheep erythrocytes stored in Alsever's solution were washed three times with 0.15 M NaCl and suspended to 3×10^8 cells ml^{-1} in phosphate-buffered saline (PBS), pH 7.2. Equal volumes of 1/1,000 diluted mouse anti-sheep erythrocyte serum were added to the red cell suspension and incubated at 37°C for 60 min. The sensitised cells were washed three times with PBS and used for the EA-rosette formation. 50 washed larvae or 10 adult worms were incubated with 5×10^4 sensitised red cells in a final volume of 1 ml hydrolysate lactalbumin Earle's glucose (HLEG) at 37°C for 30 min. After gentle resuspension, rosette formation was observed. Larvae or worms surrounded by more than five red cells on the surface were scored as positive. The intensity of the rosetting was graded from ++ (partial erythrocyte binding, >30 RBC) to +++ (totally coated with erythrocytes).

showed a partial erythrocyte binding (Fig. 1b) usually starting at the anterior end of the parasite, which becomes totally coated within 1 h of *in vitro* incubation.

The specificity of the schistosomula binding site for immunoglobulins was assessed with various heterologous proteins of an approximately similar molecular weight. Previous incubation at a concentration of 1 mg ml^{-1} for 30 min at 37°C with ribonuclease I (Worthington), cytochrome c from horse heart (type VI, Sigma) or lysozyme from egg white (grade I, Sigma) did not inhibit rosette formation, whereas inhibition was observed on incubation with immunoglobulin from different animal species (man, rabbit, mouse, rat) (Table 2). Specificity for IgG binding was similarly studied by various inhibition experiments. Incubation of schistosomula with unaggregated (100 $\mu\text{g ml}^{-1}$) or aggregated (1 mg ml^{-1}) IgG (Table 2) from different animal species (man, rat, mouse and rabbit) entirely inhibited subsequent rosette formation, whereas incubation with IgA, IgM or IgE had no inhibitory effect (Table 2). Evidence for the specific binding of IgG to a possible Fc receptor was indicated by two series of experiments.

Table 2 Specific inhibition of EA rosettes by IgG or Fc γ on skin schistosomula

Schistosomula	Sensitised sheep RBC
Non-incubated	+++
Previously incubated with:	
Human, rat, mouse and rabbit Ig	—
Human IgM, IgA, IgE (50 $\mu\text{g ml}^{-1}$)	+++
Unaggregated (100 $\mu\text{g ml}^{-1}$) or aggregated human IgG (1 mg ml^{-1})	—
Fc fragments of human IgG (40 $\mu\text{g ml}^{-1}$)	—
F(ab') ₂ fragments of rabbit anti-human IgG (10 $\mu\text{l ml}^{-1}$)	+++

In each experiment, 50 larvae were preincubated with components adjusted to appropriate concentrations with 1 ml HLEG at 37°C for 30 min and washed three times with HLEG before addition of sensitised sheep red blood cells. The intensity of the rosetting was graded as in Table 1. The purity of IgA (α I subclass) or IgM each with λ light chains was verified by the Ouchterlony technique. Fc fragments were prepared according to the method of Porter⁹ with papaine, and purified on DEAE-cellulose (0.3 M NaCl). F(ab')₂ fragments of rabbit prepared by pepsin digestion (ratio: 1/50, 10 mg ml^{-1} proteins in 0.1 M sodium acetate, pH 4.5, 24 h, 37°C) were purified by gel filtration through Sephadex G-200. The purity of fragments was verified by immunoelectrophoresis against specific antisera.

When schistosomula were incubated with Fc fragments of human IgG, total inhibition of rosette formation was observed, whereas incubation with rabbit IgG F(ab')₂ anti-human IgG did not affect erythrocyte binding (Table 2). On the other hand, no rosette formation was observed when schistosomula were incubated with ox erythrocytes sensitised with an F(ab')₂ fraction prepared by pepsin digestion from rabbit anti-ox RBC IgG (Table 3) and with an anti-ox RBC 19S fraction purified from rabbit (Table 3).

Enzyme sensitivity of the Fc receptor was also estimated by rosette inhibition experiments. Fc receptors on schistosomula were found to be trypsin resistant (trypsin TRTPCK, Worthington) (0.5 mg ml^{-1} for 30 min at 37°C) and pronase (Calbiochem) (0.5 mg ml^{-1} for 30 min at 37°C) sensitive. Results of binding of radioiodinated IgG to Fc receptors on schistosomula will be published elsewhere (G.T., in preparation).

The experiments reported here indicate the existence on schistosomula of a specific binding site for the Fc portion of IgG possessing the specificity of Fc γ receptors described on eukaryotic cells. The appearance of the above described receptors seems to follow a dynamic process during the early stages of the parasite life cycle. It was observed that although free-living

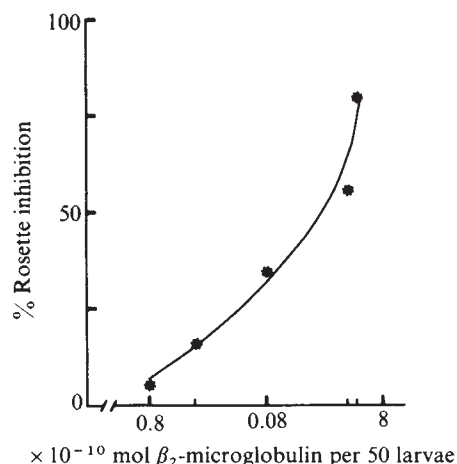


Fig. 2 Inhibitory effect of human β_2 -microglobulin on EA-rosette formation. Schistosomula collected *in vitro* after mouse skin penetration were washed three times with large volumes of HLEG. For use in the inhibition assay, 50 larvae were incubated in 1 ml HLEG with different amounts of β_2 -microglobulin with frequent mixing at 37°C for 30 min. Schistosomula were then washed in HLEG and the rosette formation was observed with sensitised sheep RBC (EA) prepared as described in Table 1. Larvae surrounded by less than five red cells on the surface were counted as negative and their percentages were calculated from at least 30 schistosomula.

cercariae did not express these surface receptors, maximum density of binding sites was observed by rosette formation on 3-h-old schistosomula collected after skin penetration. Strikingly, newly transformed schistosomula collected after mechanical tail disruption showed a progressive appearance of binding starting at the anterior end of the parasite, near the glandular apertures, and extending to the whole body within 1 h of *in vitro* incubation. This dynamic process might be related to the excretion of parasite enzymes known to occur during this period⁸. In the later life stages of the parasite, 5-d-old schistosomula and adult worms no longer express the ability to form rosettes.

The binding of IgG to the Fc receptor on eukaryotic cells is not inhibited by β_2 -microglobulin despite the homology between this molecule and the third domain of the γ chain. As histocompatibility antigens are known to bind to schistosomula³ we investigated whether β_2 -microglobulin interfered with EA rosetting. When schistosomula were first incubated with human β_2 -microglobulin (Pharmacia), total inhibition of rosette formation was observed at a concentration of 5 ng ml⁻¹ for 30 min at 37°C (Fig. 2).

The present results indicate that IgG binds to *S. mansoni* schistosomula. The affinity of the receptor for β_2 -microglobulin suggests two possibilities: (1) a receptor for IgG which, because of the phylogenetic distance between trematodes and mammals presents some unusual features and is mostly for the third domain of the γ chain, or (2), a receptor for β_2 -microglobulin

binding IgG through the homology with its third domain. In any case, the receptor might be involved in the specific binding of murine major histocompatibility antigens³ on schistosomula in addition to that of IgG. The precise relationship between these two host antigens is still under investigation.

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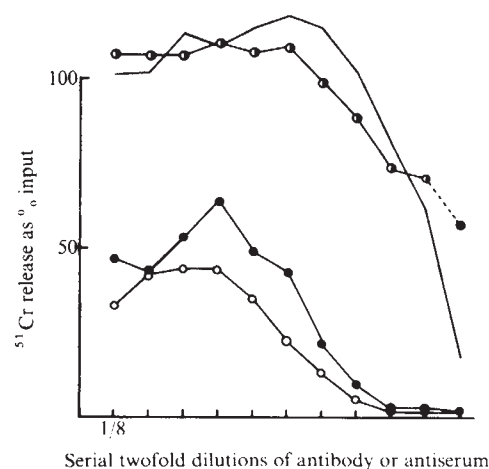
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Demonstration of MHC-specific haemolytic plaque-forming cells

IT would be convenient to be able to measure the antibody response to alloantigens of the major histocompatibility complex (MHC) at a cellular level. As MHC alloantigens are expressed on erythrocyte membranes in mice one might suppose that a conventional Jerne plaque assay with enumeration of haemolytic plaque-forming cells (allo-PFC) against a lawn of



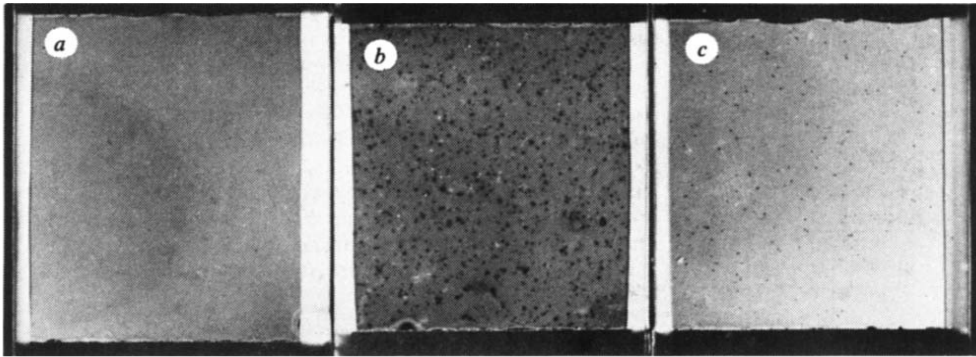


Fig. 2 Plaque formation by spleen cells from an AO rat immunised against DA lymphoid cells (AO 1; see Table 1 legend for immunisation and technical details). 5×10^5 cells per Cunningham chamber: a, No additional reagents added to the assay; b, R2/10P (HL) monoclonal anti-DA (H-1A^a) antibody added at 1/30 final; c, R3/47 (HL) monoclonal anti-DA antibody added at 1/10 final. ($\times 2$.)

suitable allogeneic erythrocytes would be straightforward. In fact such assays have proved difficult, with very few plaques being detected^{1,2}. We have found the same problem in detecting allo-PFC in the spleens of alloimmunised rats. Although alloantisera raised in appropriate strain combinations have a high titre in a direct complement dependent haemolytic assay (Fig. 1) emphasising the potential value of this convenient assay for alloantibody in the rat^{3,4}, very few allo-PFC were found in spleen cell suspensions from hyperimmunised donors. We show here that the discrepancy may be due to the homogeneity of the antibody product of a single PFC compared with the heterogeneity of serum antibody.

During a study of the haemolytic properties of monoclonal anti-rat MHC (H-1) alloantibodies generated by somatic cell hybridisation^{4,5} we observed that of six monoclonal anti-H-1A^a IgG antibodies studied none was able to lyse more than a proportion (<50%) of DA (H-1A^a) erythrocytes at saturation, whereas mixtures of appropriately chosen pairs of monoclonal antibodies would regularly lyse 100% of erythrocytes (Fig. 1). The principal factor limiting cell lysis by a single monoclonal alloantibody was determinant density on the target cell⁵. Recent data demonstrate that erythrocyte populations are heterogeneous in H-1A^a determinant density and that cells with high determinant density are lysed preferentially by a single monoclonal alloantibody (unpublished results). The determinant density dependence of lysis is radically reduced when appropriate mixtures of monoclonal antibodies are used. The definition of 'appropriate' in this context is that the two monoclonal antibodies used should bind noncompetitively to different determinants on the same target molecule. In these conditions, for reasons which are not yet clear, the two antibodies synergise strongly in lytic activity⁵.

The failure to demonstrate allo-PFC in the spleens of hyperimmune donors may therefore have been due to the fact that only a small proportion of erythrocytes was lysed by the monoclonal antibody product of each potential plaque-forming cell resulting in 'plaques' that were too faint to be seen. It therefore seemed likely that if one component of a synergistic pair of monoclonal antibodies was added to the plaque assay system in excess, clear plaque formation would occur around a cell secreting a complement fixing antibody that was not competitive with the exogenous antibody. Spleen cells taken from AO (H-1^m) rats 6 and 7 days after an intravenous boosting immunisation with DA (H-1^a) lymphoid cells showed the characteristic low but measurable allo-PFC activity in conventional Cunningham⁶ plaque chambers. When exogenous monoclonal anti-H-1A^a antibodies were added to the chambers an enormous increase in plaque count was seen (Table 1, Fig. 2). The size of the increment was a function of the monoclonal antibody added: three monoclonal antibodies probably directed against the same determinant⁵ (R2/10P, R2/15P and R3/13) were highly efficient in developing plaques. Monoclonal antibodies directed against another part of the molecule (R2/10S, R2/15S and R3/47) were less efficient. This order has been maintained (with the single exception of AO4) in all animals assayed (Table 1 and unpublished results). It was also noticeable that plaques forming in the presence of R2/10P, R2/15P and R3/13 were larger than those forming with the other antibodies (Fig. 2). It is therefore possible that the differential efficiency is a reflection of differential visibility of plaques. Alternatively, each group of antibodies might allow a distinct subset of the plaque-forming cell response to be seen (that is those producing antibody not competitive with the added monoclonal antibody) and that the subsets are regularly of different sizes.

Table 1 Demonstration of allo-plaque-forming cells using synergistic lysis with monoclonal alloantibodies

Antibody added*	Plaque-forming cells per 10^6 spleen cells						
	Unimmunised AO rats		Immunised AO rats				
	AO(N1)	AO(N2)	1	2	3	4	5
0	<0.1	<0.1	43	97	54	40	20
R2/10P (HL)	—	—	1,950	5,226	3,677	—	—
R2/15P (HLGK)	—	—	2,100	4,548	—	—	—
R3/13 (HL)	0.6	1.8	2,000	3,742	—	480	540
R2/10S (HLK)	—	—	43	742	—	—	—
R2/15S (HL)	<0.1	0.2	825	2,338	2,290	640	240
R3/47 (HL)	—	—	545	1,323	—	—	—
Anti-rat IgG†	—	—	>500	48	26	400	600

AO ♀ rats at 3–4 months old were immunised against DA spleen and lymph node cells by primary subcutaneous and intraperitoneal injection on day 0, followed by an intravenous boosting injection of DA lymphoid cells at day 142 (rat 1), day 43 (rats 2 and 3), or days 43 and 72 (rats 4 and 5). Spleen cells were taken 6 days (rats 1–3) or 7 days (rats 4 and 5) after the i.v. boost, suspended in Dulbecco's phosphate-buffered saline containing 2% fetal calf serum (DAB/FCS), and diluted to an appropriate concentration for assay. Fresh DA erythrocytes were washed in phosphate-buffered saline and diluted to a 25% suspension in DAB/FCS for use. Guinea pig serum absorbed 1:4 with DA blood cells was used as a source of C' at a final concentration of 1/10. Plaque assays were incubated in Cunningham chambers for 60 min at 37°C.

* Monoclonal antibody supernatants were used at final concentrations between 1/10 and 1/30 in the plaque-forming cell assay with the exception of R2/10S on AO 2. In this case the supernatant was concentrated 10-fold by $(\text{NH}_4)_2\text{SO}_4$ precipitation between 50% and 60% saturation and used finally at 1/10, equivalent to undiluted supernatant.

† Anti-rat IgG was a rabbit anti-rat IgG2 antiserum (JH 1/7) used at a final (optimal) dilution of 1/30. Other anti-Ig reagents were also used in some experiments. Plaques detected with anti-Ig reagents were always small and hard to score.

Synergistic lysis by monoclonal antibodies is usually a more efficient method of demonstrating allo-plaque formation than is the addition of heterologous antiglobulin antisera (Table 1). Plaques developed by anti-IgG reagents were usually small and hard to score. This low efficiency is presumably partly responsible for the variations recorded in anti-Ig plaque scores in Table 1. We have no direct evidence that the plaques seen in the synergy experiments are produced by IgG antibody. However, since the synergistic lysis phenomenon is only known by us from IgG monoclonal antibodies, and since the assays were performed relatively late (6 or 7 days) in a secondary or tertiary response it seems probable that the exogenous monoclonal antibodies do in fact reveal IgG plaque-forming cells. It may be that the small numbers of plaques seen without the addition of a synergistic monoclonal antibody represent IgM secreting cells. We do not have an IgM anti-H-1A^a monoclonal antibody but such an antibody would be expected to be less dependent on determinant density for its lytic activity. It may be that the H-1A^a specificities are particularly suitable targets for demonstrating the synergistic development of PFC by monoclonal alloantibody, as there is evidence that these specificities are unusually abundant on erythrocytes of the DA strain⁷. However, haemolytic antisera may be raised against other haplotypes³.

The phenomenon of synergistic lysis explains why only very few allo-PFC could be detected in animals producing high titre haemolytic antisera: a lytic alloantiserum is a synergistic antibody mixture.

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The *beige* mutation in the mouse selectively impairs natural killer cell function

GERM LINE mutations affecting defined cell populations are often valuable tools in elucidating the function of these cells in complex biological systems such as tumour rejection. We report here that a mutant gene in the mouse called *beige* (*bg¹*), leads to a complete and selective impairment of naturally occurring killer lymphocytes, whereas all other forms of cell-mediated lysis are apparently normal. The defective gene product may lie within the lytic pathway subsequent to tumour cell contact. Because many cell types, including natural killer (NK) cells, T cells and macrophages, may be involved in tumour resistance *in vivo*¹, these mice will provide a critical test of the hypothesis that it is NK cells which provide a first line of defence against neoplasia². It is likely that this mutant will be invaluable for further investigations in tumour immunology just as the nude mouse has been indispensable in evaluating the role of the

thymus in the development of the T-lymphoid system and the role of T cells in the rejection of tumours.

The *beige* mutation in C57Bl/6 mice occurred spontaneously in an autosomal recessive gene on linkage group XIV (ref. 3) and is thought to provide an accurate model of the Chediak-Higashi syndrome in man⁴. Humoral immunity and delayed-type hypersensitivity are normal and the chief cause of mortality is an increased susceptibility to infection⁵ and a lymphocytic infiltration which may be malignant⁶.

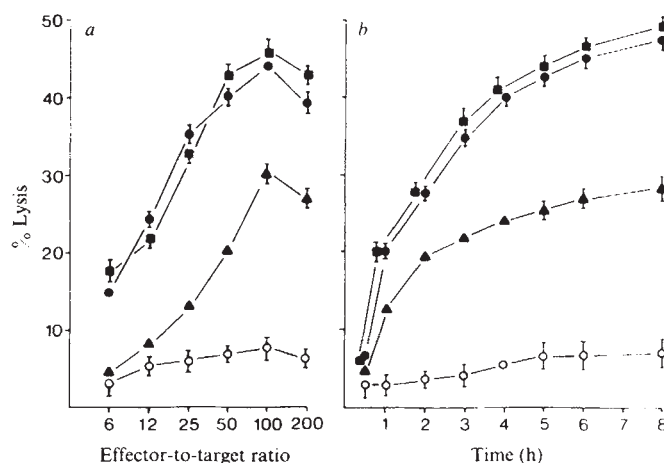


Fig. 1 The absence of NK-mediated cytotoxicity in *beige* mice. Nylon wool column-passed spleen cells from 4-week-old mice were incubated with ⁵¹Cr-labelled YAC tumour cells at various effector-to-target ratios in a 4-h assay¹¹ (a) or for various periods of time at a 100/1 effector/target ratio (b). ▲, A/Sn; ○, C57Bl/6, *bg¹/bg¹*; ●, *+/bg¹* littermates; ■, age-matched *+/+* controls. Values represent the mean % specific lysis ± s.e. in quadruplicate wells.

To investigate NK cell function in mutant mice, spleen cells were passed over nylon wool columns, a procedure which enriches for NK cells (Fig. 1). Compared with one of the lowest responder strains known (A/Sn), it can be seen that the homozygous *beige* mutants (*bg¹/bg¹*) were completely deficient in their ability to lyse YAC cells, a Moloney lymphoma which is the most sensitive NK target yet described. The heterozygous littermate controls (*+/bg¹*) responded as well as the wild type (*+/+*). The low response of the *bg¹/bg¹* mice was not altered by prolonged incubation times or high effector-to-target ratios. In additional experiments (unpublished observations) the absence of NK cytotoxicity was apparent in all the NK-bearing lymphoid organs (spleen, lymph node, bone marrow and peripheral blood) and at all points in the life cycle whether tested against YAC or other NK-sensitive targets (RBL-5, MPC-11, 136-6, P-52, Molt-4). In addition, interferon or interferon-inducing agents (poly I:C, tilorone) failed to augment lysis in *beige* mice (submitted for publication) whereas these agents greatly augmented NK function in low responder strains such as A/Sn⁷ and in very young or old mice of the same strain (Gidlund, personal communication). Therefore, the deficient NK response in *beige* mice cannot be accounted for by an altered tissue distribution, age-dependent maturation or a shift in target selectivities. It was then relevant to consider if the defect in cytotoxicity was selective for NK cells or whether it involved a general failure of all forms of cell-mediated lysis.

As shown in Table 1 (lines 4-6), cytotoxic T lymphocytes generated by alloimmunisation of *+/bg¹* or *bg¹/bg¹* mice *in vivo* or by one-way mixed lymphocyte cultures (MLC) *in vitro* were equally capable of lysing P815 cells (a completely NK-insensitive target). In addition, spleen cells from *bg¹/bg¹* mice alloimmunised *in vivo* responded to secondary challenge in MLC cultures by mounting a strong response on day 3. Peak responses in primary MLC cultures were not obtained until days 5 or 6. Further experiments revealed identical kinetics of cytotoxic

Table 1 The selectivity of the NK defect

Effector cell*	Generation	Target†	Maximum lysis	Lytic units per 10 ⁶ ‡
1 +/bg ^J NK	None	YAC	53 (100/1)§	10.1 ± 0.8
bg ^J /bg ^J NK	None	YAC	5 (100/1)	0.0 ± 0.0
2 +/bg ^J ADCC	None	CRBC	45 (100/1)	1.8 ± 0.2
bg ^J /bg ^J ADCC	None	CRBC	48 (100/1)	2.0 ± 0.1
3 +/bg ^J ADCC	None	P815	25 (50/1)	8.3 ± 0.4
bg ^J /bg ^J ADCC	None	P815	2 (50/1)	0.0 ± 0.0
4 +/bg ^J CTL	<i>In vivo</i>	P815	55 (100/1)	8.3 ± 0.6
bg ^J /bg ^J CTL	<i>In vivo</i>	P815	47 (100/1)	7.8 ± 1.1
5 +/bg ^J CTL	1° MLC	P815	60 (25/1)	41.4 ± 2.7
bg ^J /bg ^J CTL	1° MLC	P815	64 (25/1)	50.2 ± 3.8
6 +/bg ^J CTL	2° MLC	P815	66 (25/1)	86.5 ± 4.9
bg ^J /bg ^J CTL	2° MLC	P815	80 (25/1)	99.1 ± 6.0
7 +/bg ^J CTL	Con. A	P815	85 (100/1)	7.8 ± 2
bg ^J /bg ^J CTL	Con. A	P815	70 (100/1)	5.0 ± 1
8 +/bg ^J Macrophage	<i>In vitro</i>	P815	30 (4/1)	33.5 ± 2.3
bg ^J /bg ^J Macrophage	<i>In vitro</i>	P815	25 (4/1)	28.7 ± 2.1

Lines 1, 2, 3: spleen cells from untreated mice were tested in a 4-h ⁵¹Cr-release assay against normal YAC targets (Moloney lymphoma from A/Sn, H-2^{kd}) or P815 cells (DBA/2 mastocytoma, H-2^{dd}) and chicken erythrocytes treated with a predetermined optimum concentration of specific antibody (1/80 A.BY × C57Bl/6 F₁ anti-A/Sn and 1/200 mouse anti-CRBC, respectively). P815 and CRBC targets treated with normal serum or untreated yielded < 2% lysis. Line 4: mice were injected intraperitoneally on day -10 with 30 × 10⁶ P815 cells (passage 25 *in vitro*) and pooled spleens were assayed in a 2.5-h ⁵¹Cr-release assay. Lines 5, 6: spleen cells from untreated mice or mice immunised *in vivo* with P815 (line 4) were cultured with mitomycin C-treated DBA/2 spleen cells at a 5/1 responder/stimulator ratio. Primary (1°) and secondary (2°) cultures were collected on days 5 and 3, respectively, and assayed in a 2.5-h test. Non-stimulated control cultures yielded < 15% lysis of P815 and stimulated cultures failed to lyse RBL-5 cells (H-2^{db}). Line 7: mice were injected intravenously at -24 h with 250 µg Con A and spleen cells were assayed in a 16-h ⁵¹Cr-release test in the presence of 10 µg ml⁻¹ Con A. Con A did not affect target cell survival and was necessary for maximum lysis *in vitro* as described previously⁸. Line 8: bone marrow cells were cultured as previously described and on day 3, non-adherent cells (promonocytes) were removed and cultured with lymphokine derived from the supernatants of 24 h, Con A-stimulated C57Bl/6 spleen cells⁹. The 'activated' macrophages which adhered to glass were cultured for 16 h with ⁵¹Cr-labelled P815 target cells. Non-activated control cultures yielded < 5% lysis.

* Cytotoxicity by effectors designated as CTL was inhibited over 90% by anti-θ serum and guinea pig complement whereas NK cells were inhibited < 10%. Neither CTL nor NK was affected by pretreatment with carbonyl iron and a magnet. Incorporation of heat-aggregated mouse gamma globulin (1–500 µg ml⁻¹) into the cytotoxic assay blocked 70% of ADCC against chicken erythrocytes (CRBC) but had no effect on lysis by NK cells, CTL or ADCC against P815. All responses of age-matched +/+ mice were similar to those of heterozygous littermate controls, +/bg^J, and therefore the wild-type values have been omitted for clarity.

† All targets were maintained by continuous *in vitro* culture except CRBC.

‡ A lytic unit was defined as the number of effector cells required for 50% maximum lysis and was calculated from parallel effector titration curves over the entire range of lysis from 0 to maximum.

§ The values given represent the mean % lysis in quadruplicate wells at the effector/target ratio given in brackets. These levels of lysis were designated as the maximum attainable, as they occurred on the plateau of effector titration curves.

T-lymphocyte generation in MLC cultures and the kinetics of target cell lysis in the cytolytic assay also overlapped (unpublished data). In addition, the normal level of lysis in *beige* mice by T cells activated with concanavalin A (Con A) indicates that the total pool of cytotoxic T cells is normal, as this technique is thought to measure all polyclonally distributed killer T cells regardless of their specificity⁸. These results strongly suggest that T cells from *beige* mice are capable of exerting normal cytolytic function.

Tumour cell destruction by other cell types also seemed normal in *beige* mice (Table 1, lines 8, 9). Bone marrow-derived macrophages activated *in vitro* with a lymphokine⁹ lysed P815 cells, as did peritoneal macrophages when activated with lipopolysaccharide¹⁰ (unpublished observation). Antibody-dependent cell-mediated cytotoxicity (ADCC) against antibody-coated chicken erythrocytes (CRBC) was also normal in *beige* mice (Table 1, line 2). As ADCC against CRBC in the mouse is mediated largely by Fc⁺, adherent cells^{11,12}, this population seems to be normal. In addition, the killing capacity of monocytes and granulocytes may also be normal in *beige* mice, as these effectors are involved in human ADCC against CRBC¹³. ADCC against tumour cells, however, is mediated by different lymphocytic effectors called K cells¹. As shown in Table 1, line 3, the function of these cells is completely deficient in the homozygous *beige* mice; this provides the strongest evidence that murine K cells and NK cells are identical, as suggested by Ojo and Wigzell¹². These workers showed that antibody-coated, NK-insensitive P815 cells competed with non-antibody-coated, ⁵¹Cr-labelled YAC cells in a cytolytic assay¹². However, murine NK and K cells directed against tumour cells do not possess readily detectable Fc receptors, as direct lysis of antibody-coated P815 cells is not inhibited by aggregated gamma globulin, as reported earlier¹¹ and confirmed here (Table 1). It is possible, therefore, that non-antibody-dependent contact with a target

triggers the expression of Fc receptors in the NK cell which can then kill by either an antibody-dependent or antibody-independent mechanism, depending on (1) the availability of antibody on the target cell and (2) the NK sensitivity of the target.

The detailed nature of the NK defect in *beige* mice is not known, but may lie within the lytic machinery of the cell rather than at the level of population size or recognition receptors. As shown in Table 2, nylon-passed spleen cells from *bg^J/bg^J* mice were completely normal in their capacity to bind to YAC targets. We have previously shown that this assay is a reliable measure of the frequency of NK cells in a mixed population^{14,15}. The target-binding lymphocytes from *beige* mice were selective,

Table 2 The frequency of target-binding cells in *beige* mice

Genotype	Preincubation of effectors*	% Target-binding cells
C57Bl/6 (+/+)	None	20.4
	NK-TS	8.1
C57Bl/6 (+/bg ^J)	None	19.8
	NK-TS	9.2
C57Bl/6 (bg ^J /bg ^J)	None	21.3
	NK-TS	7.9
A/SN	None	7.6
	NK-TS	3.4

Nylon wool column-passed spleen cells from age and sex matched mice were labelled with fluorescein and incubated with a 10-fold excess of YAC cells. The number of lymphocytes binding to YAC was then determined by counting 500 cells in an ultraviolet microscope¹⁴. Additional spleen cells were preincubated for 1 h at 4°C with 1 µg of 140 K glycoprotein isolated from YAC membranes and purified by SDS-polyacrylamide gel electrophoresis¹⁶. The inhibition of subsequent binding to intact YAC was dose dependent and was not observed when similar concentrations of purified H-2^{kd} antigen was used. NK-TS, natural killer cell-target structure, a glycoprotein isolated from YAC cells¹⁶.

as they could be inhibited by preincubation with solubilised and purified NK target antigens from YAC but were not inhibited by H-2 antigen prepared in the same manner, as previously documented in other strains¹⁶. Further experiments are necessary to localise the defective gene product in the lytic pathway subsequent to target cell contact.

In conclusion, we have described a genetic impairment in the lytic machinery of NK cells which totally prevents these cells from initiating antibody-dependent or antibody-independent cytotoxicity of tumour cells. This defect was selective for NK cells, as lysis by immune T cells, macrophages and cells participating in ADCC against a non-tumour target was normal. Additional experiments, designed to test oncogenesis and spontaneous tumour development in these mutant mice, will provide a critical test of the hypothesis that NK cells are involved in surveillance against neoplasia.

The data showing normal macrophage killing in *beige* mice has recently been confirmed and extended by Marie-Luise Lohmann-Matthes.

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Amyloid resistance in A/J mice is determined by a single gene

WIDESPREAD tissue deposition of the unique fibrous protein amyloid occurs in a variety of human diseases¹. 'Secondary' amyloidosis arises as a complication of chronic infectious or inflammatory disease and an identical syndrome can be induced in mice by repeated injections of casein². Little genetic work has been done in this area, probably because development of amyloidosis is often variable, even with a standard induction regimen, and results are inconsistent³. Furthermore, because of the many complex immunological aberrations occurring during the

amyloid induction period⁴, the various experimental manipulations which can affect it⁴, and the wide range of induction times seen in different murine strains², one would expect that multiple genes and alleles would influence amyloidogenesis. Nevertheless, the discovery of the uniquely resistant A strain⁵ suggested the possibility of isolating at least part of the genetic basis for amyloid susceptibility or resistance. We now report a genetic analysis of amyloid induction in two inbred strains, CBA/J and A/J, differing markedly in susceptibility to amyloidosis⁵. We show for the first time that a single gene confers resistance on the A/J strain. Our results imply that genetic factors may also define subpopulations of chronic disease patients who are at greater risk from amyloid disease.

Table 1 No. of mice in each group resistant to a 6-week period of amyloid induction by azocasein

Group	No. tested	No. resistant
CBA/J	20	0
(CBA × A) _F ₁	20	0
A/J	20	20
F ₁ × A/J	43	19

Although we have generally used casein in our experimental protocols, the more potent, predictable amyloidogen azocasein⁶ was chosen for these experiments. We find that five subcutaneous injections per week of 10% azocasein induces amyloid in 100% of the highly susceptible CBA/J strain within 2 weeks. Induction time has been consistent with different batches of azocasein and in different experiments, in contrast to our experience with casein. F₁ hybrid mice were produced by mating CBA/J females with A/J males. Preliminary work established that although all CBA/J mice had amyloidosis after 2 weeks of azocasein, 3-4 weeks were required to achieve a 100% incidence in F₁ hybrids. In contrast, the earliest appearance of amyloid in A/J mice was at 7 weeks, with a less than 30% incidence after 8 weeks of injections. We tentatively concluded that although the F₁ heterozygous mice had a slightly prolonged amyloid induction period, the marked resistance of the A/J strain was a recessive trait.

For the definitive genetic experiment, females of the F₁ hybrid group were mated to A/J males. CBA/J, F₁ hybrid, backcross and A/J mice were subjected to serial azocasein injections, five per week, and congo red-stained spleen sections were then scored for the presence or absence of amyloid. F₁ hybrid mice were studied after 2 or 4 weeks of injections. An induction period of 6 weeks was selected for backcross mice, as this is well beyond the period required to induce a 100% incidence of amyloid in F₁ hybrid mice, but too soon to produce amyloid in any A/J mice. Groups of A/J mice were studied after 6, 7 or 8 weeks of injections. Under this protocol we expected that only those backcross mice with resistance similar to that of the A parent would remain free of amyloid. The percentage of amyloid-resistant mice in the backcross group would allow an estimate of the number of genes involved in determining resistance. The results are shown in Table 1. In agreement with preliminary experiments, 100% of CBA/J mice had amyloid when studied at 2 weeks and 100% of F₁ hybrids had amyloid at 4 weeks. After 6 weeks, no amyloid was found in A/J mice, although one of six A/J animals had amyloid after 7 weeks (not shown), demonstrating that 6-7 weeks is the threshold for amyloid induction in the A strain in these laboratory conditions. In the backcross group, 19 of 43 animals were resistant to 6 weeks of azocasein injections. This is close to the 50% incidence of amyloid resistance expected if it were the expression of a single gene, and significantly greater than the 25% incidence expected for a trait requiring two genes.

In contrast to other groups, there was a different incidence of amyloid in males and females of the backcross group; 13 of 22

females but only 6 of 21 males were resistant (data not shown). In addition, male spleens contained histologically more amyloid than female spleens. A simple explanation for this is that males of this group in particular demonstrated considerably more fighting than other groups of mice. As wounds from fighting alone are a major cause of amyloidosis in male mice⁷, and as inflammation correlates with amyloidosis⁸, the biting and clawing of injection sites observed in these mice would be expected to increase amyloid production. Therefore, the incidence of resistance in 13 of 22 female mice probably represents the 'true' incidence.

We are not proposing that a single genetic locus alone governs susceptibility to amyloid. The genetic data presented here, however, demonstrate that a single gene with a major influence on amyloidogenesis can account for the extreme degree of resistance seen in the A strain, compared with CBA mice.

The mechanism of amyloid resistance remains unknown, but several possibilities are suggested by previous work. The inducing agent casein has been demonstrated to be a polyclonal B-cell activator in the susceptible CBA strain⁹. Although genetic analysis of this B-cell response was not carried out, the genetics of B-cell response to lipopolysaccharide, another amyloid-inducing agent, has been extensively studied; a single gene has been shown to account for variation in macrophage and B-cell responsiveness to this agent¹⁰. The role of B-cell activation in amyloid production is not known, but differences in B-cell function during amyloid induction in CBA and A mice have been demonstrated¹¹. Thus, a single gene determining B-cell responses to azocasein is plausible by analogy to other systems.

A very different hypothesis is suggested by recent work on the cellular degradation of the AA protein, a major amyloid component, and SAA protein, its serum precursor¹². Although three different patterns of breakdown of SAA were observed, one particular pattern of enzymatic breakdown was found in all amyloid patients but only some normal controls¹². If a single enzyme system involved in degradation of SAA protein is indeed a critical factor in amyloidogenesis, then a single genetic locus could very well have a major influence on amyloid susceptibility, as suggested by the data presented here.

The present data do not allow us to choose between these or other hypotheses accounting for the unusual amyloid resistance of the A/J strain mouse. However, the demonstration that a single genetic locus may be the basis for the resistance offers the hope of elucidating one of the critical steps in the pathogenesis of amyloid.

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Histamine-coated particles generate superoxide (O_2^-) and chemiluminescence in alveolar macrophages

WE have recently shown that histamine bound to red cells forms 'rosettes' with various guinea pig leukocytes and that these 'histamine receptors' are particularly well expressed on the alveolar macrophage¹. As the lung is a major site of histamine storage and high concentrations of histamine are present in the sputum in certain respiratory diseases such as chronic bronchitis and asthma^{2,3}, we have performed experiments to determine whether free histamine, or histamine bound to particles, interacts with its membrane receptor to initiate the respiratory burst in alveolar macrophages. Activation of the respiratory burst in phagocytic cells is achieved by a number of agents, both soluble

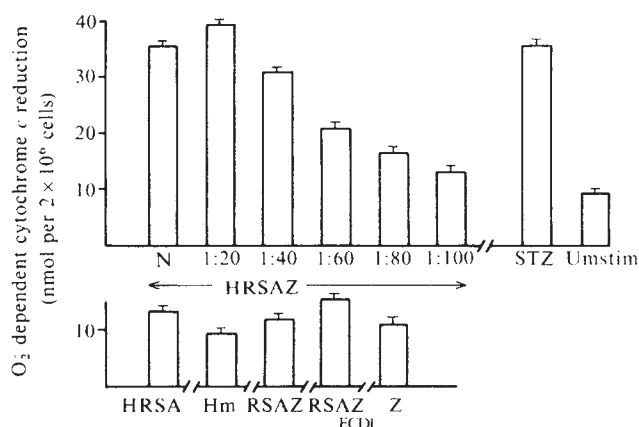


Fig. 1 The generation of O_2^- by alveolar macrophages activated with histamine bound to zymosan (HRS AZ). Guinea pig alveolar macrophages, obtained by broncho-tracheal lavage, were purified by centrifugation on Ficoll-Hypaque, washed three times with the balanced salt solution (BSS⁺⁺) described previously⁴ and adjusted to a concentration of 2.4×10^6 cells ml^{-1} . This procedure was repeated if there were any contaminating granulocytes. These procedures gave alveolar macrophages of 90-95% purity which were free of granulocytes and red cells, the contaminating cells being virtually all lymphocytes. The cells (0.8 ml) were incubated with 0.1 ml of the various agents indicated or with BSS⁺⁺ to obtain the resting or unstimulated (Unstim.) values. Following addition of cytochrome c (100 nmol in 0.1 ml) in the presence or absence of superoxide dismutase (4.2 nmol in 50 μ l), the mixtures were incubated at 37°C for 30 min, transferred to an ice-bath and centrifuged at 1,000g for 5 min. The O_2^- activity was measured as cytochrome c reduction (calculated from the decrease in absorbance at 550 nm on addition of potassium ferricyanide as described previously⁹) using a Pye Unicam SP 1800 UV spectrophotometer. Any reduction occurring in the presence of superoxide dismutase was subtracted from these values. Histamine dihydrochloride (1.4 g) was conjugated to 200 mg rabbit serum albumin (HRS A) with 1.2 g of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (ECDI) contained in 20 ml phosphate buffered saline (PBS) and extensively dialysed as described by Kedar and Bonavida¹⁰. For neat (N) HRS AZ, zymosan (10 mg) was incubated with 1 ml of HRS A for 45 min at 37°C, washed twice and resuspended in 1 ml of PBS at pH 7.2. Dilutions of HRS A from 1 in 20 to 1 in 100 were also incubated with 10 mg of zymosan and similarly incubated and washed. For HRS A alone, zymosan was omitted; for RS AZ, histamine and ECDI were omitted and histamine was omitted in the preparation of RS AZ_{ECDI}. Serum treated zymosan (STZ) was prepared by incubating 1 ml fresh guinea pig serum with 10 mg zymosan and incubating and washing as for HRS AZ. Hm, histamine dihydrochloride (10^{-3} mol l^{-1}), Z, zymosan (10 mg in 1 ml PBS). When unstimulated cells or cells with treatments were incubated in the presence of superoxide dismutase, values obtained were less than 2.00. Each test was performed in duplicate and the results represent the mean $\pm$ 1 s.e.m. of four experiments.

and particulate, many of which bind to the plasma membrane (for example, particles opsonised with complement⁴) through cell surface receptors. This suggests that there may be a close association between 'membrane recognition' of certain exogenous stimuli and the enzyme which initiates the primary oxygen-consuming reaction (also thought to be membrane bound and probably 'NADPH oxidase'⁵). In the experiments reported here superoxide anion formation (O_2^-) and chemiluminescence have been measured as these are recognised features of the respiratory burst of phagocytic cells⁵.

O_2^- generation by alveolar macrophages was measured by dismutase-inhibitable cytochrome *c* reduction. When histamine in free solution was incubated with purified alveolar macrophages, free of other phagocytic cells, the amounts of O_2^- formed were no more than those obtained with resting (unstimulated) cells. In contrast, when histamine was bound to particles such as zymosan (a yeast cell-wall extract) large amounts of O_2^- were generated. The binding of histamine (H) was achieved by first coupling the amine to rabbit serum albumin (RSA) (see Fig. 1) and then allowing this HRS conjugate to adsorb on to the particle. Histamine coupled in this way is known not to be substantially altered chemically⁶. Also, we were able to show that bound histamine retained its biological activity in terms of its capacity to contract the isolated guinea pig ileum. The results shown in Fig. 1 were with zymosan although several other particles which absorbed the coupled histamine also generated O_2^- . These included a cross-linked dextrose gel (Sephadex G-10), a bead-formed agarose gel (Sephacrose 4B), a cross-linked polystyrene polymer (Amberlite XAD-2) and latex (polystyrene) beads. By themselves, or following addition of RSA alone, these particles did not generate appreciable amounts of O_2^- , indicating that the observed effect was independent of the chemical properties of the carrier and was a result of the histamine conjugated to RSA. The O_2^- formed was directly proportional to the quantities of HRS conjugate offered to the particles (Fig. 1). Zymosan which had adsorbed a 1 in 20 dilution of HRS generated as much O_2^- as zymosan incubated with fresh serum. Serum (as a source of complement) coated zymosan (STZ) is known to be particularly effective in initiating the respiratory burst in many phagocytic cells, including the alveolar macrophage⁷. When alveolar macrophages were incubated with the various controls shown in Fig. 1, the amount of O_2^- generated was similar to that in unstimulated cells. These results indicate that the observed effect was attributable to bound histamine and not to the zymosan alone or zymosan coated with the carrier protein in the presence (RSAZ_{ECDI}) or absence (RSAZ) of the coupling reagent (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide). Of these controls RSAZ_{ECDI} gave the highest values but this was not significantly different from RSAZ, HRS, Hm or Z.

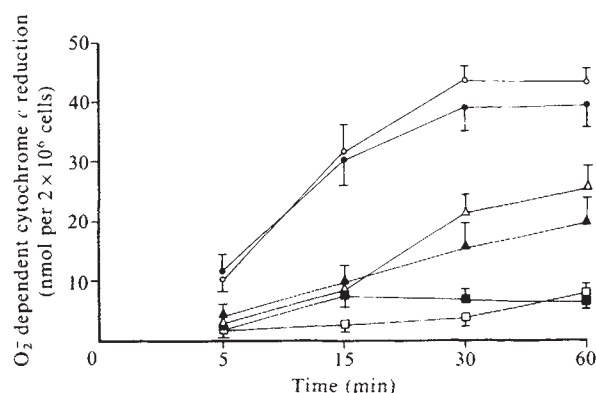


Fig. 2 The time course of generation of O_2^- by alveolar macrophages activated with histamine bound to zymosan (HRSZ). STZ (○), HRSZ (1 in 20) (●), RSAZ (△), HRS (▲) and Hm (■) (10^{-3} mol l^{-1}) were prepared as in Fig. 1. □, unstimulated (resting) cells. Each time point was performed in duplicate and results represent the mean $\pm$ s.e.m. of four experiments.

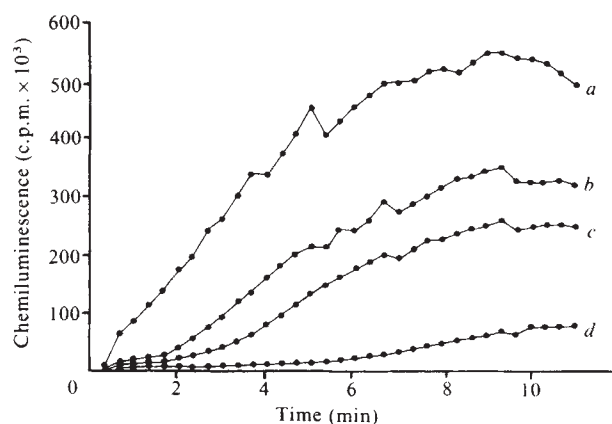


Fig. 3 Chemiluminescence by alveolar macrophages activated with histamine bound to zymosan (HRSZ). HRSZ (0.1 ml) was incubated with 0.5 ml of 0.2 mM solution of luminol (Sigma) dissolved as described¹¹, and contained in BSS⁺⁺. Two million alveolar macrophages contained in 0.4 ml were added to this mixture and the counting commenced immediately. Experiments were performed in polypropylene minivials (Searle) contained in glass scintillation vials with pre-bored screwcaps (Koch-Light) which were pre-heated at 37°C for 18 h. All manipulations were performed in the dark at 37°C. Counts were recorded at room temperature in a Beckman LS-250 liquid scintillation counter, operating in the 'out of coincidence' mode¹², using a minimal iso-set module adjusted to detect approximately the lower third of the tritium energy spectrum. The counting mode was set to record infinite sequential, 0.2 min total count readings at a present error of 0.2%. HRSZ dilutions: a 1:10; b, 1:20; c, 1:40; d, 1:80. The figure is representative of six experiments. Peak luminescence with the controls, HRSZ + superoxide dismutase, RSAZ_{ECDI}, RSAZ, HRS, RSA, Z, Hm (10^{-3} mol l^{-1}) and unstimulated gave values < 73 c.p.m. $\times 10^3$. STZ gave peak luminescence of 617 c.p.m. $\times 10^3$.

The time course of O_2^- production by alveolar macrophages stimulated with histamine bound to zymosan by the RSA conjugate (HRSZ) is shown in Fig. 2. This was compared with STZ, RSAZ, free histamine, the histamine conjugate in the absence of particles (HRS) and the amounts formed by unstimulated macrophages. Maximal O_2^- generation with HRSZ and STZ was achieved within 30 min whereas with RSAZ and HRS the amounts detected were much less and did not 'plateau', even after 1 h. The relative differences between O_2^- produced by alveolar macrophages following activation by the various agents and the amounts produced by unstimulated cells were similar to those obtained in Fig. 1. These experiments (Figs 1 and 2) indicate that bound histamine is comparable to bound complement in its capacity to initiate the respiratory burst in alveolar macrophages and that the effects are both dose- and time-dependent.

We were also able to show that O_2^- production by bound histamine was an H_1 - and not an H_2 -receptor dependent effect. This was determined in seven experiments in which the alveolar macrophages were preincubated for 30 min with the appropriate drugs at various dilutions. The H_2 -receptor antagonists, burimamide and metiamide, at doses of 5×10^{-5} , 2×10^{-5} and 0.8×10^{-5} mol l^{-1} , produced insignificant inhibition of O_2^- production, the values being $8.3\% \pm 3.0$, $9.5\% \pm 2.9$, $10.5\% \pm 3.8$, and $3.4\% \pm 1.7$, $2.2\% \pm 1.6$, $8.4\% \pm 4.3$, respectively. In contrast, the H_1 -antagonists, chlorpheniramine and mepyramine at the same concentrations, gave linear dose-dependent inhibitions of $22.3\% \pm 3.8$, $13.8\% \pm 3.6$, $10.7\% \pm 4.4$ and $24.4\% \pm 5.0$, $19\% \pm 4.8$, $12.8\% \pm 3.3$, respectively. The inhibition produced by the highest doses of the H_1 -receptor antagonists were highly statistically significant ($P < 0.005$). The drugs had no significant effect on the resting values of O_2^- production or on O_2^- generated by STZ indicating that HRSZ probably initiates the respiratory burst by the H_1 -dependent histamine receptor that we previously described using the 'rosette technique'¹.

Chemiluminescence, which also accompanies the respiratory burst of phagocytic cells⁵, was initiated by histamine bound to zymosan. As shown in Fig. 3 this effect was both time- and dose-dependent. This experiment was repeated five times and gave similar results. Chemiluminescence induced by HRSZ or STZ was completely inhibited by superoxide dismutase indicating that light emission by these cells was closely associated with O_2^- production.

These results suggest that there may be a direct relationship between histamine and lung macrophages. We have established that histamine, when bound to a number of different particles, initiated the respiratory burst in these cells. The response, like the 'histamine rosettes' described previously¹, was H_1 -receptor dependent.

Although histamine alone was without effect it is known to bind to a number of plasma proteins ('histaminopexy'⁸) and so our observation may be of significance both physiologically, in the regulation of oxygen-dependent microbial killing by lung macrophages, and in disease states such as asthma and chronic bronchitis^{2,3}. In these latter situations high sputum concentrations of histamine may adversely influence the well recognised defensive role of the alveolar macrophage and so contribute to respiratory infections.

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Anti-inflammatory steroids induce biosynthesis of a phospholipase A_2 inhibitor which prevents prostaglandin generation

ASPIRIN prevents prostaglandin (PG) generation by directly inhibiting the cyclo-oxygenase enzyme responsible for PG biosynthesis¹⁻³. In addition, there is now conclusive evidence that anti-inflammatory steroids can also prevent PG generation⁴⁻¹³. Unlike the aspirin-like drugs, steroids have no anti-cyclo-oxygenase activity but exert their action by preventing the release from phospholipids of the fatty acid substrates required for PG biosynthesis^{4-9,12,13}. We have shown that stimulation of thromboxane A_2 (TXA₂) release by agents such as histamine, 5-hydroxytryptamine and rabbit aorta contracting substance-releasing factor (RCS-RF) (but not arachidonic acid) is inhibited

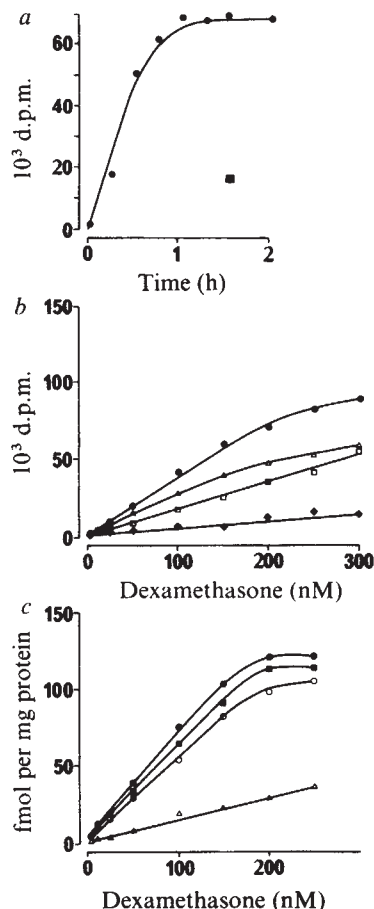


Fig. 1 Male guinea pigs (250-300 g) were killed by cervical dislocation and exsanguination, and lungs were removed and washed in ice-cold saline and then homogenised in 10 ml Tris buffer (pH 7.5, 20 mM containing 2 mM $CaCl_2$, 1 mM Na_2MoO_4 and 1 mM $MgCl_2$). The homogenate was centrifuged at 100,000 g for 90 min at 2°C. The supernatant was retained on ice until use, (usually within 2-3 h of preparation). Protein content was estimated by the Biuret method. The binding assay¹⁷ used reaction sets of 0.1 ml Tris buffer (20 mM pH 7.5), 0.3 ml cytoplasmic fraction, 0.05 ml unlabelled steroid or saline control and 0.05 ml 3H -dexamethasone, incubated for 2 h at 4°C. Unbound dexamethasone was adsorbed by adding acid-washed Norit charcoal (5 mg) and vortex mixing for 20 s. Charcoal was removed by centrifugation (10,000 g for 10 min). Supernatant (0.3 ml) was removed for estimation of bound dexamethasone using liquid scintillation counting. **a**, Total binding of dexamethasone by lung supernatant as a function of time. 3H -dexamethasone (20 pmol) was used for this experiment; binding was maximal by 90 min; also shown is the uptake in the presence of 10^{-5} M unlabelled dexamethasone. **b**, Total binding of dexamethasone by lung supernatant (ordinate) as a function of the free steroid (abscissa). Curves represent one of four experiments. Binding varied between lungs, and although results were clear at <300 nM dexamethasone, the binding above this concentration became erratic and difficult to reproduce. The 'control' curve is derived from samples containing only 3H -dexamethasone. The ' 3H background' curve is derived from samples containing no cytoplasmic protein. The other curves show displacement of the 3H -dexamethasone from the receptor by cortisone (10^{-5} M) and 10^{-5} M unlabelled dexamethasone; this concentration gave a maximal displacement of the labelled drug. The protein concentration was 6.2 mg ml^{-1} . **c**, 'Specific' binding of dexamethasone is defined here as that binding resistant to the addition of 10^{-5} M unlabelled drug, and is calculated by subtracting the binding in the presence of 10^{-5} M dexamethasone from the binding found in its absence. Figures are expressed as fmol dexamethasone bound per mg cytoplasmic protein. Also shown is the displacement of the binding curve by 10^{-7} M dexamethasone, 10^{-6} M hydrocortisone and 10^{-5} M cortisone. Results are calculated from an experiment similar to that shown in **b**. The binding varied between experiments and a range of 100-400 fmol dexamethasone per mg cytoplasmic protein was observed in the four experiments carried out. ●, Control; ■, dexamethasone; △, cortisone; ○, hydrocortisone; ♦, 3H background.

by anti-inflammatory steroids, and that their potency in this action closely parallels their anti-inflammatory activity^{12,13}. Furthermore, their mechanism of action involves the inhibition of phospholipase A_2 activity, and thus of arachidonate release within the lung^{12,13}. In several other tissues, the mechanism of steroid hormone action depends on the combination of the

steroid with a cytosolic receptor protein, the translocation of this drug-receptor complex to the nucleus and the initiation of protein biosynthesis¹⁴⁻¹⁶. We now show that similar events occur in the guinea pig perfused lung before inhibition by steroids of phospholipase A₂ activity (and thus TXA₂ generation). We have discovered a steroid-induced factor which mimics the anti-phospholipase effects of these anti-inflammatory agents.

Dexamethasone was used for most of the experiments, although results were confirmed with triamcinolone and hydrocortisone. Dexamethasone was used because it is a potent inhibitor, available as the soluble sodium phosphate salt (Decadron, Merck), and can be obtained in labelled form ([1,2(n)³H]dexamethasone, 39 Ci mmol⁻¹, Radiochemical Centre). We used this tritiated steroid to look for a steroid receptor protein in lung cells. The method was that of Baxter and Tomkins¹⁷ (see Fig. 1) which shows that there is a time-dependent, saturable 'specific' binding of labelled dexamethasone by the crude cytosol fraction of lung homogenate. This binding was reduced or prevented by unlabelled dexamethasone (10⁻⁷-10⁻⁵ M), higher concentrations (10⁻⁶-10⁻⁴ M) of the weaker anti-inflammatory steroid hydrocortisone, or by the 'anti-glucocorticoid' corticosterone (10⁻⁶-10⁻⁵ M), which binds to other steroid receptors¹⁸. The binding capacity of the cytosolic supernatant was abolished by pretreatment with papain (100 µg ml⁻¹ protein), or by heating the preparation to 40 °C for 60 min. The bound steroid could be removed from the binding protein by vigorous shaking with *n*-heptane. The radioactivity thus liberated co-chromatographed with authentic dexamethasone on TLC (developing solvent, chloroform:methanol:water, 9:1:0.1, v/v), indicating that no metabolism of dexamethasone had occurred. These results show that dexamethasone is bound to a receptor protein in one or more cell types in the lung.

In the second series of experiments, we investigated the activity of actinomycin D (an inhibitor of RNA but not protein biosynthesis)¹⁹, cycloheximide (an inhibitor of protein and RNA biosynthesis)²⁰ and puromycin (an inhibitor of protein biosynthesis)²⁰ on the steroid-induced suppression of phospholipase activity. Guinea pig isolated lungs were prepared²¹, and perfused with oxygenated Krebs' solution (37 °C) at 10 ml min⁻¹. First, we measured the release from the perfused lung of TXA₂ in response to stimuli (such as RCS-RF) which act through phospholipase activation¹². TXA₂ release was detected by a superfused rabbit aortic strip, and the TXA₂ generated by a standard stimulus was compared with that produced when a known amount of sodium arachidonate was injected through the lung. Infusion of steroids through the lung selectively abolishes stimuli which act through phospholipase activation without affecting arachidonate stimulation. Thus, the ratio between the two responses is a measure of phospholipase activity^{12,13}.

Second¹³, a specifically labelled phospholipid (2-[³H-oleoyl]phosphatidylcholine) was injected through the lung (with a small amount of [¹⁴C]-oleic acid to act as an internal standard). Free oleic acid liberated by the action of phospholipase A₂ within the pulmonary vascular bed is measured by liquid scintillation counting of the extracted effluent. The hydrolysis of this phospholipid is suppressed by steroids and the results closely parallel those obtained by bioassay^{12,13}. Figure 2a shows that in the untreated lungs, dexamethasone (1 µg ml⁻¹) markedly reduced phospholipase activity as estimated by the release of TXA₂ or by hydrolysis of the radioactive phosphatide. This inhibition was reversed after the steroid infusion was discontinued, and responses had fully recovered after 1 h (not shown). Figure 2b-d shows that cycloheximide (2 µg ml⁻¹), actinomycin (1 µg ml⁻¹) or puromycin (5 µg ml⁻¹) either abolished or reduced the inhibitory effect of the steroid. Indeed, a potentiation of phospholipase activity was sometimes detected. The blockade of the inhibitory activity of steroid by inhibitors of RNA or protein synthesis implies that the action of the steroids depends on a transcriptional as well as a translational step. Cycloheximide pretreatment of the lung did not diminish the number of steroid receptors in cytosolic preparations.

Having demonstrated that corticosterone displaced dexamethasone from the cytoplasmic receptor, we tested its effect in an intact perfused lung. Corticosterone (25 µg ml⁻¹) given with or during an infusion of dexamethasone (1 µg ml⁻¹) reduced the inhibitory effect of the latter on phospholipase activity (as estimated on both biological and radiochemical assays) by up to 85%. Corticosterone itself had no intrinsic activity at these concentrations.

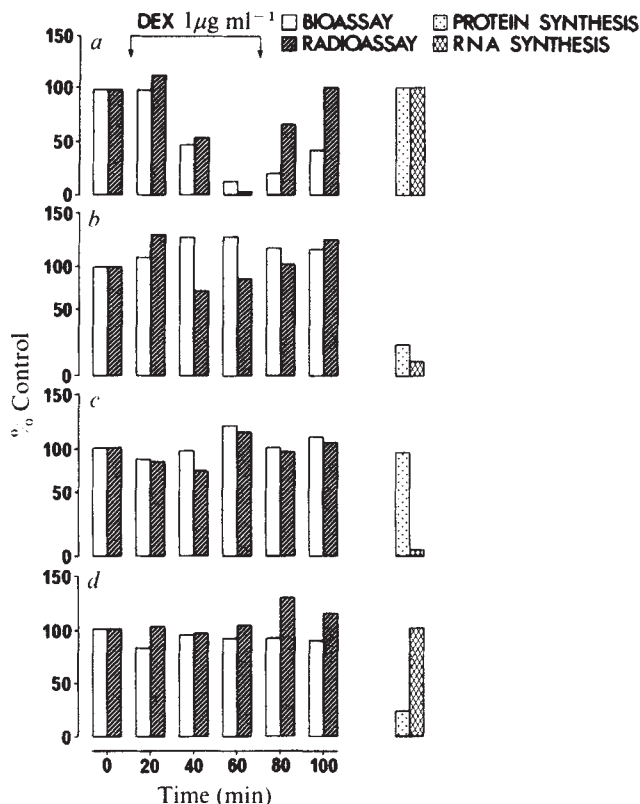


Fig. 2 The effect of dexamethasone (1 µg ml⁻¹) on lung phospholipase activity and the release of TXA₂, and the abolition of this action by protein and RNA synthesis inhibitors. The guinea pig isolated perfused lungs were prepared, and the bio- and radioassays carried out as described^{12,13}. All data have been reduced to the same format and are expressed as a per cent change from the control response. For the bioassay the anti-phospholipase activity was calculated as a decrease in the ratio between the release of TXA₂ by a standard dose of RCS-RF and a standard dose of arachidonic acid. Phospholipase activity is expressed as the per cent change in hydrolysis (pmol oleic acid released) occurring on injection through the lung. The data shown here represent one of 3-5 experiments. Protein and RNA synthesis was estimated by infusing 0.150 µCi [¹⁴C]-lysine or 12.0 µCi [³H]-uridine into a perfused lung over 1 h. Some lungs received saline only, others actinomycin (1 µg ml⁻¹), cycloheximide (2 µg ml⁻¹) or puromycin (5 µg ml⁻¹). After 1 h the lung was chopped with scissors and homogenised in a glass homogeniser. The proteins and nucleic acids were precipitated with 5 vol ice-cold 1 M perchloric acid and the precipitate was sedimented in a centrifuge. This precipitate was washed twice more with 5 vol 0.5 M perchloric acid and the precipitate was sedimented in a centrifuge. This precipitate was then washed with 5 vol ethanol: ether (1:1) and the final pellet solubilised with concentrated NaOH. The radioactivity was estimated by liquid scintillation counting after neutralisation with acetic acid, and the protein content was estimated by the Biuret reaction. Results are expressed as per cent changes from control d.p.m. per mg protein. a, Control; b, +2 µg ml⁻¹ cycloheximide; c, +1 µg ml⁻¹ actinomycin; d, +5 µg ml⁻¹ puromycin.

Clearly, steroids act in the perfused lungs in much the same way as they act to produce metabolic effects in other tissues. Could this effect be produced by stimulating the biosynthesis of an inhibitory factor, perhaps a polypeptide? We have now demonstrated the presence of such a factor using perfused, cycloheximide-treated lungs which are thus resistant to the inhibitory action of steroids. First, effluent from a perfused lung (generator lung) was re-oxygenated and pumped directly through another perfused lung (test lung) which also received

cycloheximide ($2 \mu\text{g ml}^{-1}$) to render it insensitive to steroids. Anti-inflammatory steroids were infused either through the generator lung (TL) or into the effluent leaving the generator lung (IE). Thus, the only difference in the effluent perfusing the test lung was whether or not the steroid had passed through the first lung. Bioassay or radiochemical assays were as described earlier.

When steroids were infused into the test lung (Fig. 3) they did not affect phospholipase activity. When steroids were infused into the generator lung, however, phospholipase activity of the test lung (estimated by either technique) was greatly reduced. Similar results were obtained when the steroids were perfused TL first and then changed to IE. In this case the depressed phospholipid hydrolysis and responses to RCS-RF returned when the steroids were infused IE.

Second, two lungs were perfused simultaneously, one received steroid TL the other IE. The only difference in the perfusates was that in one case the steroid had perfused through the lung and in the other it had gone directly into the effluent. After storage at 4°C overnight or for longer periods of time, the flasks of effluent were re-oxygenated and alternately perfused through a lung receiving cycloheximide. Some results obtained with this technique are shown in Fig. 4. When perfused with fresh Krebs' solution, or the effluent containing steroid-infused IE, there was no inhibition of phospholipase activity, but when perfused with effluent from steroid-treated lungs the basal phospholipid hydrolysis was depressed or abolished and the responses to RCS-RF attenuated. Return to normal occurred when the steroid was omitted from the ordinary Krebs' solution or when effluent was used from lungs where steroids were infused IE.

No metabolism of dexamethasone occurred in the lungs, as infusing the tritiated drug, extracting it from the Krebs' solution with *n*-heptane and analysing the product by TLC showed that all the radioactivity co-chromatographed with authentic dexamethasone. Triamcinolone acetonide and hydrocortisone hemisuccinate gave similar effects.

We conclude that the anti-phospholipase action of steroids is induced by a mechanism essentially similar to that mediating their other metabolic actions. First, the steroid combines with a receptor located in the cell cytoplasm of one or more cell types in the lung. The amounts of steroid required to saturate the receptors in the lung (10–20 pmol per mg protein) are higher than those reported for some other tissues (see refs 17, 22), and

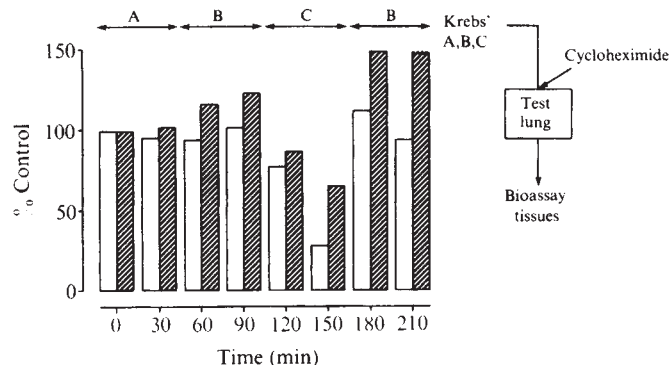


Fig. 4 Steroids induce the biosynthesis of a stable anti-phospholipase factor. The assay was with a single perfused lung receiving cycloheximide ($2 \mu\text{g ml}^{-1}$) to prevent the action of steroids. The lung is perfused with one of three batches of Krebs' solution: a, normal Krebs' solution; b, the lung effluent to which dexamethasone ($1 \mu\text{g ml}^{-1}$) had been added; c, the effluent from a lung in which dexamethasone ($1 \mu\text{g ml}^{-1}$) had passed through the lung. Only during an infusion of solution c was there a fall in the phospholipase activity of the recipient lung. The steroid-induced factor was stable in Krebs' solution at 4°C for at least 7 but less than 14 d. □, Bioassay; ▨, radioassay.

the actual binding (fmol per mg protein) is much less. Perhaps there are more receptors per mg lung protein, or they have a much lower affinity than those of other tissues studied. Scatchard plots of the binding data were non-linear, perhaps indicating the presence of more than one receptor population. Certainly, combination with the receptor is important for the action of steroids, as corticosterone displaced labelled dexamethasone from the receptor and blocked the inhibitory action of this steroid in the perfused lung system. Combination of corticosterone with the steroid receptor may form an inactive complex¹⁸.

Our experiments do not show whether combination of the steroid with the receptor is followed by translocation of this complex to the nucleus. However, the subsequent genetic expression does occur in lung tissue and this involves transcription as well as translation, for inhibitors of RNA as well as of protein biosynthesis block the steroid effect. The disparity of chemical structure and site of action suggests that these inhibitors exert their effect in this system by an action on the RNA/protein synthetic machinery. Interestingly, actinomycin D blocks the anti-inflammatory effect of steroids *in vivo*²³. (During preparation of this paper, Danon and Assouline²⁴ reported findings similar to some of ours in rat renal papillary tissue.)

Clearly, in perfused lungs, steroids induce the synthesis of a factor which blocks phospholipase A_2 and this factor can be transferred from one perfused lung to another. Presumably, it is a peptide or protein, as its biosynthesis is prevented by cycloheximide. The biosynthesis of this factor may be crucial to the anti-inflammatory effect of steroids. We have observed the close relationship between the anti-phospholipase activity and the anti-inflammatory activity of corticosteroids¹². There is considerable evidence linking the generation of prostaglandins with inflammation, pain and fever (see ref. 25), and several other products of fatty acid oxidation such as 12-hydroxy-5,8,14-eicosatetraenoic acid²⁶ or malondialdehyde²⁷ could also be pro-inflammatory or cytotoxic. Furthermore, slow-reacting substance A may be derived from arachidonic acid^{28,29}. The non-steroid aspirin-like drugs block the generation of cyclo-oxygenase products^{1,30} but because the substrate fatty acids are rate limiting (see refs 12, 13), the anti-inflammatory steroids can prevent the biosynthesis of a whole cascade of lipid mediators, so that the steroid-induced anti-phospholipase factor could be important in controlling the inflammatory response. If this is so, steroids would not have an anti-phospholipase effect in cells which do not contain steroid receptors.

Our discovery that the action of steroids in this tissue depends on the continued biosynthesis of protein and RNA also means

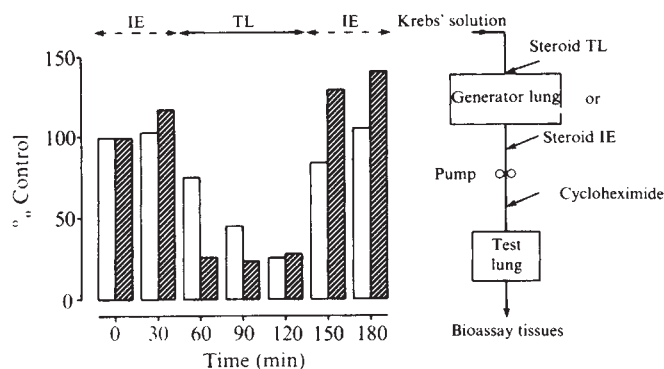


Fig. 3 A factor induced by steroids is released into the effluent of the lung and can have an anti-phospholipase effect in a second lung. Two lungs were perfused in series as shown. The re-oxygenated effluent from the 'generator lung' was pumped into the test lung which also received cycloheximide ($2 \mu\text{g ml}^{-1}$) to prevent the action of steroids. Dexamethasone ($1 \mu\text{g ml}^{-1}$) was infused either directly into the effluent from the first lung (IE) or first through the generator lung (TL). During the equilibration period the steroid was infused IE and there was no change in the activity of the enzyme; however, when infused TL there was an immediate fall in phospholipase activity of the second lung showing that a factor was formed in the first lung which had an anti-phospholipase effect in the second. When the steroid was again perfused IE the phospholipase activity returned to control values. Similar results were obtained in five other experiments. □, Bioassay; ▨, radioassay.

that these drugs do not exert their effect by 'membrane stabilisation'. We do not yet understand how the steroid-induced factor inhibits the phospholipase and what (if any) other pharmacological actions this factor has. Some phospholipases require 'activation' before catalysis can proceed; in some cases this involves the removal of a peptide protecting the active centre³¹. One effect of the steroid-induced factor could be to mimic the action of this peptide. Whatever the mechanism of action, the discovery that the anti-phospholipase activity of steroids is mediated by a soluble 'second messenger' is likely to contribute to the understanding of the anti-inflammatory action of these drugs.

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Stimulatory effect of vanadate on $(\text{Na}^+ + \text{K}^+)$ -ATPase activity and on ^3H -ouabain-binding in a cat heart cell membrane preparation

VANADATE has been reported to inhibit $(\text{Na}^+ + \text{K}^+)$ -ATPase activity in membrane preparations from dog kidney¹ and in red cells². This inhibition, which occurs in the nanomolar concentration range in the presence of high Mg^{2+} concentrations and in the micromolar range at physiological Mg^{2+} concentrations^{2,3} is specific as other ATPase enzymes are not affected in these conditions¹. Therefore vanadate has been suggested to be 'an ideal specific regulator' of $(\text{Na}^+ + \text{K}^+)$ -ATPase¹. We have shown⁴ that vanadate has a distinct positive inotropic effect in cat papillary muscle. It has been claimed that the positive inotropic action of cardiac glycosides, which are specific inhibitors of $(\text{Na}^+ + \text{K}^+)$ -ATPase activity^{5,6}, is associated with the

inhibition of that enzyme. If that is so, vanadate would be expected to inhibit $(\text{Na}^+ + \text{K}^+)$ -ATPase, in a concentration-dependent manner, in parallel with its effect on force of contraction. To test this hypothesis, we measured the effect of vanadate on $(\text{Na}^+ + \text{K}^+)$ -ATPase activities and on ^3H -ouabain-binding in identical conditions in a membrane preparation taken from cat hearts that were also used for experiments on contraction. We report here, however, that vanadate may stimulate $(\text{Na}^+ + \text{K}^+)$ -ATPase activity transiently and ^3H -ouabain-binding permanently in these conditions.

After the papillary muscles had been dissected from the cat hearts⁴, remaining ventricular tissue was frozen quickly and kept at -60°C for up to 4 weeks until further use. After thawing, $(\text{Na}^+ + \text{K}^+)$ -ATPase (EC 3.6.1.3.) was prepared from 12 hearts as described by Pitts and Schwartz⁷ but without glycerol treatment. Enzyme activity was determined by the coupled optical assay in an ATP-regenerating system⁸. It was measured as 0.32 U mg^{-1} membrane protein; 97% of total ATPase activity could be inhibited by ouabain (10^{-4} M). One enzyme unit is defined as $1 \mu\text{mol ATP hydrolysed per min at } 37^\circ\text{C}$. Protein was determined by the method of Lowry *et al.*⁹.

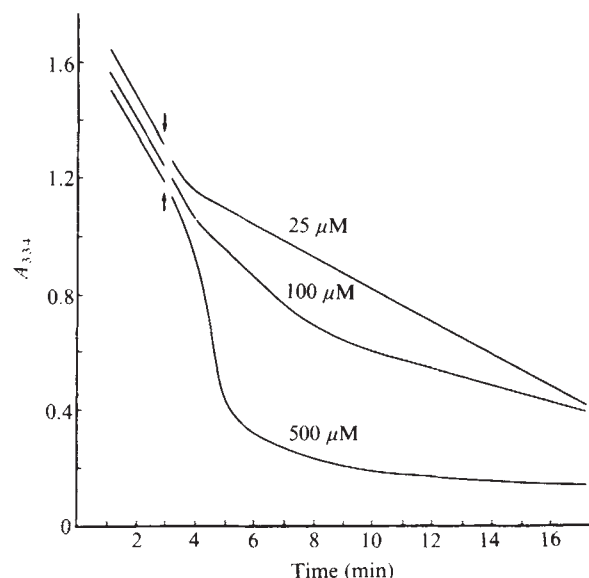


Fig. 1 Stimulatory and inhibitory effect of vanadate on $(\text{Na}^+ + \text{K}^+)$ -ATPase activity. Compounds were dissolved in H_2O daily. Incubation medium was the same as that used for the determination of force of contraction⁴ and contained (mM): NaCl , 136.9; KCl , 5.4; CaCl_2 , 1.8; MgCl_2 , 1.05; NaH_2PO_4 , 0.42; glucose, 5.5 (buffered with NaHCO_3 to pH 7.4); total volume was 2 ml. Enzyme protein (1 mg) was incubated in this medium for 120 min at 37°C in each test tube with different concentrations of NH_4VO_3 (Merck, Darmstadt). Then 0.2 ml of this mixture was assayed rapidly for ATPase activity in the coupled optical assay⁸, followed by continuous recording photometrically for 20 min at least. The time of addition of NH_4VO_3 is marked by arrows.

The effects of NH_4VO_3 on $(\text{Na}^+ + \text{K}^+)$ -ATPase activity were measured as described in the legend to Fig. 1. A typical photometric recording is shown in Fig. 1. Enzyme activity is followed continuously by the degradation of NADH at 334 nm. The disappearance of NADH is related stoichiometrically to the hydrolysis of ATP in the coupled optical assay⁸. Soon after addition, NH_4VO_3 in a final concentration of $25 \mu\text{M}$ inhibited $(\text{Na}^+ + \text{K}^+)$ -ATPase about 65%. Higher concentrations initially inhibited the enzyme appreciably less ($100 \mu\text{M}$) or even stimulated it above control values for several minutes. We refer to this as 'transient initial stimulation'. For instance, $500 \mu\text{M}$ stimulated the enzyme 180% for about 3 min (Fig. 2). But after about 5 min it caused progressive inhibition. This rather strange effect

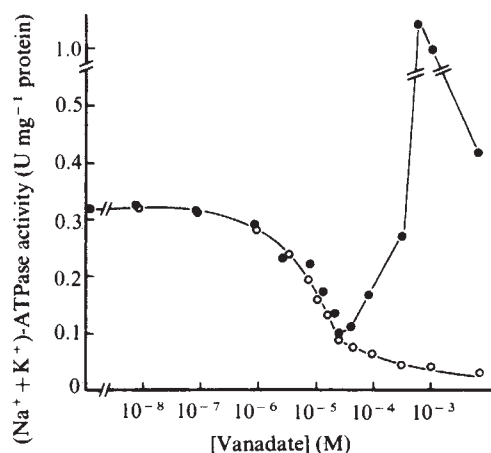


Fig. 2 Concentration-dependent effect of vanadate on $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$ activity. Cat heart ventricular cell membranes (1 mg protein) were preincubated for 120 min in medium, as described in the legend to Fig. 1, to which was added increasingly large amounts of NH_4VO_3 , total volume 2 ml each. After 120 min, 0.2 ml of this medium containing enzyme protein was assayed rapidly for ATPase activity in the coupled optical assay with an ATP-regenerating system⁸ at the same vanadate concentrations as during preincubation. ●, The highest initial ATPase activity, measured usually after 3 min (Fig. 1); ○, The lowest activity measured within 20 min. Vanadate concentrations above $30 \mu\text{M}$ cause an initial transient stimulation, followed by inhibition. Similar results were obtained in three other experiments.

was not due to the action of NH_4VO_3 on the enzymatic reaction in the coupled optical assay (phosphoenolpyruvate, pyruvate kinase, lactic dehydrogenase, NADH) because the incubation medium alone (without $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$ -containing cell membranes), or with heat-denatured cardiac cell membranes, did not respond in this way to the addition of NH_4VO_3 in the respective concentrations.

In a second set of experiments cat heart ventricular membranes were preincubated for 120 min in the same incubation medium used for the determination of force of contraction in the cat papillary muscles⁴ (as described above), with and without increasingly large concentrations of NH_4VO_3 (0.005 – $2.500 \mu\text{M}$) at 37°C . After 120 min an aliquot of membranes was assayed for $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$ activity by the coupled optical assay⁸ (Fig. 2). The results reveal a concentration-dependent inhibition of $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$ activity up to $25 \mu\text{M}$ NH_4VO_3 , half-maximal at $5 \mu\text{M}$. An initial transient stimulation was seen at the same concentrations as in the absence of preincubation, starting at $50 \mu\text{M}$ and becoming maximal at 500 – $1,000 \mu\text{M}$. Further experiments showed that preincubation had no effect unless ATP was present, thus suggesting that vanadate is bound

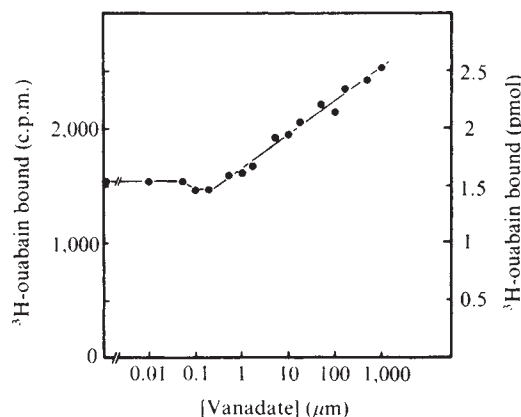


Fig. 4 Effect of vanadate on ^3H -ouabain-binding to cat cardiac cell membranes. Cat heart ventricular cell membranes (0.17 mg protein) were incubated for 120 min in medium, as described in the legend to Fig. 1, to which was added 3 mM ATP (Boehringer, Mannheim), $6 \text{ p mol } ^3\text{H}$ -ouabain (specific activity $14.4 \text{ Ci mmol}^{-1}$, lot no. 500-183, New England Nuclear, Dreieichen) and the indicated concentrations of NH_4VO_3 . ^3H -ouabain bound to the membranes was determined after rapid filtration (Whatman GF/C glassfibre filters). The radioactivity on the filters was determined by liquid scintillation counting¹⁰. Unspecific binding of ^3H -ouabain to the membranes was determined after addition of unlabelled 10^{-4} M ouabain. It was always less than 3% of total ^3H -ouabain-binding and was subtracted from the amount of total bound radioactivity. Similar results were obtained in two other experiments.

The reasons for the apparent discrepancy between our results and reports that vanadate exclusively inhibits $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$ from various sources may be either the use of the phosphate determination method^{1,2,11} (which does not facilitate continuous monitoring of the hydrolysis of ATP) or the determination of enzyme activity by the coupled optical assay after equilibrium had been established ('after... the time curve became linear')³. It is possible that the initial transient stimulation of $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$ activity by vanadate has been overlooked by other investigators. This effect was not unique to the cat heart enzyme, however. Similar results were obtained with $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$ preparations from horse and human hearts (not shown). Furthermore, in accordance with previous reports³, the concentrations of vanadate needed to inhibit the enzyme were much lower in the presence of high concentrations of Mg^{2+} (10 or 20 mM). NH_4Cl alone did not affect ATPase activity in the conditions used. Further addition of 20 mM NH_4Cl caused no measurable change in the effects of vanadate on enzyme activity. Results were the same with NH_4VO_3 or Na_3VO_4 , which had the same positive inotropic action.

The biological significance of the transient stimulatory and steady-state inhibitory effects of vanadate on $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$ activity is not clear. Transient stimulation occurred at

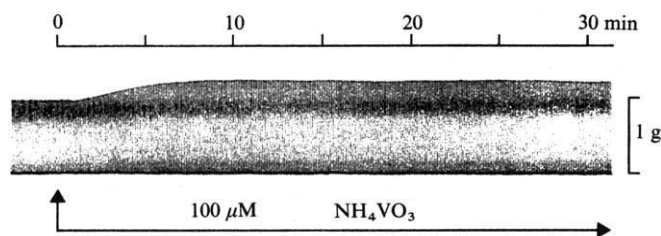


Fig. 3 Time course of the positive inotropic effect of $100 \mu\text{M}$ NH_4VO_3 in an electrically driven cat papillary muscle, mounted for the recording of isometric contractions, as described previously⁴. NH_4VO_3 was added at zero time and was present throughout the experiment. Frequency of stimulation, 0.2 Hz ; temperature, 35°C , extracellular Ca^{2+} concentration, 1.8 mM ; preparation diameter 0.8 mm .

the same concentration range as did the inotropic effects⁴. Both began rather rapidly (1–2 min) after the application of NH_4VO_3 , but the former lasted only for a few minutes, whereas the latter remained virtually stable for at least 30 min, which was the longest time investigated (Fig. 3). Perhaps the stimulatory effect triggers the inotropic effect. Of course both the stimulation and the inhibition of the enzyme might be an artefact due to the disruption of the cardiac cell membrane. However, the transient stimulatory effect of vanadate concentrations that also increase the force of contraction might be related to the reported stimulation of $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$ by very low but positive inotropic concentrations of cardiac glycosides^{6,12,13}. That effect is controversial⁶, however, although recent studies have suggested stimulation of the active transmembranous transport of cations after addition of low but positive inotropic concentrations of cardiac glycosides^{14–16}.

It is well established that the enzyme protein containing $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$ activity spans the cell membrane and specifically binds cardiac glycosides with high affinity on the outside of the cell membrane⁶. Cardiac glycosides supposedly exert their positive inotropic effect after binding to the enzyme^{6,17}. Thus, it seemed reasonable to examine the effect of vanadate on specific ^3H -ouabain-binding to $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$ of the cat ventricular cell membrane preparation in the same conditions. Figure 4 shows that vanadate at concentrations greater than $0.5\ \mu\text{M}$ stimulates ^3H -ouabain-binding in a concentration-dependent manner. Further experiments (not shown) demonstrated that this effect was due exclusively to an increased affinity of the binding sites for ouabain as the number of binding sites stayed constant. Cantley *et al.*^{3,18} have shown, however, that vanadate does not interact with ouabain-binding sites but rather with the ATP-binding sites of the enzyme at the inner side of the cell membrane. Thus the increased binding of ouabain to $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$ seems to be an indirect effect of vanadate in certain conditions.

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Thyrotropin-releasing hormone: central site of action in antagonism of pentobarbital narcosis

THYROTROPIN-RELEASING HORMONE (TRH) is capable of antagonising pentobarbital narcosis, and this effect is independent of its hypophysiotropic function^{1,2}. The potency of intraventricular injections^{2,3} and the localisation of TRH in nerve terminals from both hypothalamic and extrahypothalamic sites^{4,5}, suggest that TRH may function as a neuromodulator in the central nervous system (CNS)⁶. Although TRH antagonism of pentobarbital has been behaviourally and electroencephalographically^{7,8} characterised, the underlying neuroanatomical substrate remains unknown. In an attempt to identify the central substrate mediating this effect, we have used intracerebral microinjection of TRH into specific brain loci. Brain sites were chosen to represent large neuroanatomical regions—as cholinergic activation has been hypothesised to mediate pentobarbital antagonism by TRH, structures belonging to the cholinergic activating system⁹ were examined, and we also examined medially located brain regions that Wei *et al.*¹⁰ found to be most active at inducing TRH-mediated shaking. While a number of sites were found responsive at 500 ng of TRH, the septal region proved exceptionally sensitive by responding significantly at only 5.0 ng TRH.

Male Sprague-Dawley rats (280–330 g at the time of surgery) were individually housed under a 12-h light-dark cycle. The animal was allowed at least 7 days to recover, following surgery, and a minimum of 5 days between administrations of drug. Using pentobarbital anaesthesia (50 mg per kg body weight, intraperitoneally), each animal was bilaterally implanted with guide cannulas (26 gauge) in 2 of 19 brain loci (four cannulas per animal). Implantations were made to a depth of 1 mm above designated injection sites, and, where applicable angled to avoid ventricular penetration. Injections were made with a 33-gauge needle connected to a 1- μl Hamilton syringe by polyethylene tubing (PE-10). A small air bubble was introduced into the tubing to give a visible indication of fluid movement. Total injection volumes were $0.5\ \mu\text{l}$ per hemisphere administered over a 30-s period, and 10 s were allowed to elapse before removal of the needle. Test animals were injected intraperitoneally with 40 mg per kg pentobarbital. At 20 min after loss of righting reflex, TRH (Abbott) dissolved in 0.9% saline, or an equal volume of saline alone, was bilaterally injected. After intracranial injection, the animal was placed on its side, and return of righting reflex recorded when the animal had righted itself three times in 60 s. Cannula location was verified histologically. Before intracardiac perfusion with 10% formalin, a

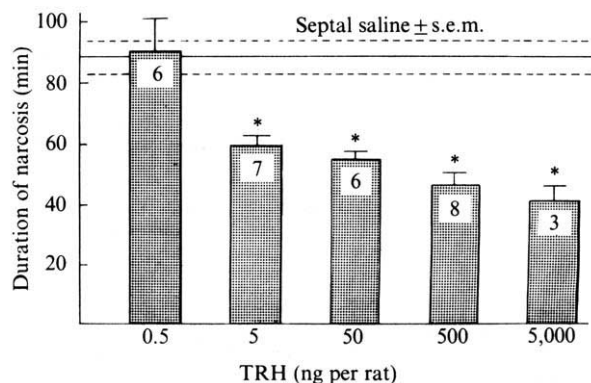


Fig. 1 Dose-response curve for intraseptal TRH antagonism of pentobarbital narcosis. The number of animals tested at each dose is shown in each column. * $P < 0.001$ compared with septal saline, two-tailed Student t -test.

Table 1 Effect of intracerebral TRH on the duration of pentobarbital narcosis measured from loss of righting reflex

Brain region	Dose (μ g)	Stereotaxic coordinates anterior	lateral	(mm) $\dagger$ vertical	Narcosis duration§ (min)
Saline in septum $\dagger$	—	7.4	0.3	1.5	87.9 $\pm$ 5.1 (14)
Somatosensory cortex	5.0	8.6	1.5	5.6	103.8 $\pm$ 10.6 (5)
Lateral hypothalamus	5.0	6.8	3.0	-2.0	95.7 $\pm$ 15.3 (6)
Amygdala	5.0	6.0	4.8	-2.1	90.5 $\pm$ 11.0 (6)
Caudate	5.0	7.8	3.0	1.0	79.5 $\pm$ 8.0 (6)
Cerebellum	5.0	-5.3	0.5	-1.0	78.6 $\pm$ 5.8 (8)
Lateral preoptic area	5.0	7.6	2.0	-1.9	75.5 $\pm$ 5.8 (6)
Parafascicular nuc.	5.0	3.6	1.3	-0.3	65.7 $\pm$ 5.0 (7)*
Parafascicular nuc.	0.5				71.4 $\pm$ 7.5 (5)
Dorsal hippocampus	5.0	3.2	2.8	2.0	63.5 $\pm$ 5.1 (6)*
Dorsal hippocampus	0.5				77.3 $\pm$ 3.0 (6)
Mesencephalic reticular nuc.	0.5	1.8	2.0	-2.2	69.7 $\pm$ 2.9 (7)
Interpeduncular nuc.	0.5	1.6	0.3	-4.8	63.1 $\pm$ 4.7 (7)*
Medial hypothalamus	0.5	5.0	0.5	-3.0	59.9 $\pm$ 1.6 (8)**
Accumbens	0.5	9.2	1.5	0.0	56.7 $\pm$ 3.2 (7)**
Substantia nigra	0.5	1.8	2.0	-4.0	56.6 $\pm$ 4.2 (7)**
Thalamic PVG	0.5	3.6	0.3	-2.1	55.6 $\pm$ 4.0 (7)**
Locus coeruleus	0.5	-1.8	0.6	-3.0	55.6 $\pm$ 3.3 (5)*
Locus coeruleus	0.01				75.8 $\pm$ 2.3 (5)
Mesencephalic PVG	0.5	0.8	0.5	-0.7	53.0 $\pm$ 2.9 (8)**
Mesencephalic PVG	0.01				68.4 $\pm$ 4.7 (7)
Medial preoptic area	0.5	7.9	0.5	-1.7	50.7 $\pm$ 5.0 (7)**
Medial preoptic area	0.01				65.8 $\pm$ 5.8 (5)
Medial thalamus	0.5	5.2	0.7	0.0	48.9 $\pm$ 2.7 (8)**
Medial thalamus	0.01				67.8 $\pm$ 4.3 (6)
Septum	0.5	7.4	0.3	1.5	45.9 $\pm$ 3.4 (8)**
Septum	0.01				59.6 $\pm$ 2.6 (7)**

$\dagger$ The coordinates are from the atlas of Pellegrino and Cushman²³ and are respective to the interaural line with the upper incisor bar elevated to 5 mm.

$\ddagger$ Duration of septal saline narcosis is not statistically different from that of rats receiving pentobarbital alone ($\bar{x}$ = 99.5 $\pm$ 6.9 min; N = 10; P > 0.1). All significance values are with respect to septal saline, determined with a two-tailed Student t -test.

$\S$ Mean $\pm$ s.e.m.; no. of determinations shown in parentheses.

* P < 0.01; ** P < 0.001.

small lesion was placed 1 mm below each guide cannula by passing 1 mA of anodal current for 3 s. Brains were frozen, 50- μ m sections made at the lesion site, and sections stained with cresyl violet.

Doses of TRH for each site were chosen on the basis of preliminary data obtained using whole-brain doses of 500 ng. Sites not demonstrating a significant decrement in the duration of narcosis at 500 ng, as compared with animals receiving only pretreatment with 40 mg per kg pentobarbital, were further examined with 5- μ g doses of TRH. Even with 5 μ g of TRH several areas proved to be insensitive, including the amygdala, cerebellum, somatosensory cortex, caudate, lateral hypothalamus, and lateral preoptic area (P > 0.01) (Table 1). In contrast, the hippocampus and parafascicular nucleus did demonstrate TRH sensitivity to 5- μ g doses. However, considering the magnitude of the dosage and the proximity of these sites to the ventricular system and/or sites demonstrating TRH sensitivity at 500 ng, the possibility that TRH may be diffusing from the injection site to more sensitive areas should be considered. That many areas remained insensitive to TRH, even at a dose of 5 μ g, is in contrast to the results of Carino *et al.*¹¹ who found both the cortex and cerebellum of the rabbit to be sensitive to a dose of 1 μ g. A possible explanation for the differences observed might be that rabbits and rats respond differently. Alternatively, Carino *et al.* used injection volumes of

2 μ l, and diffusion of TRH to active sites could also be an explanation.

As in the investigation of TRH-induced shaking by Wei *et al.*¹⁰, we found that the more medially located brain regions were most sensitive to TRH antagonism of pentobarbital narcosis. The moderate sensitivity observed in the present study with the relatively lateral nucleus accumbens and substantia nigra is an exception. The most sensitive area was the septal region. The dose-response curve for TRH antagonism of pentobarbital narcosis in the septum demonstrates that significant antagonism occurred with a dose as low as 5.0 ng (Fig. 1). While several sites responded significantly at 500 ng TRH, when the five most sensitive areas, including the septum, medial preoptic area, mesencephalic periventricular grey (PVG), locus coeruleus, and medial thalamus, were examined at a dose of 10 ng, only the septum differed significantly from septal saline injections (P < 0.01). This finding indicates at least a twofold higher sensitivity of the septum over the next most sensitive areas and implicates it as the most important site of action in TRH antagonism of pentobarbital narcosis. This evidence of marked sensitivity is supported by numerous data, including immunohistochemical¹² and chromatographic¹³ identification of TRH in the septal region, the demonstration of calcium-dependent release of immunoreactive TRH from septal synaptosomes in the presence of elevated potassium concentrations¹⁴, and the demonstration by Winokur and Beckman¹⁵ that septal neurones respond to iontophoretic TRH. Furthermore, Burt (ref. 16 and personal communication) has found that the greatest concentration of high-affinity TRH-binding sites in calf and sheep extrahypothalamic brain tissue was located in the septum and nucleus accumbens.

TRH has often been associated with modulating cholinergic transmission. This is based on investigations indicating that anti-muscarinic compounds can effectively antagonise TRH-induced arousal of pentobarbital-narcotised animals¹, and studies by Yarbrough¹⁷ demonstrating TRH potentiation in the excitatory response of cortical neurones to iontophoretic acetylcholine (ACh). Moreover, it has long been known that pentobarbital increases cortical and hippocampal ACh levels¹⁸, and Schmidt¹⁹ has shown that TRH will reverse this effect of pentobarbital on ACh levels in both the hippocampus and cortex. The importance of the septum in central cholinergic pathways is demonstrated by the observation that this region is heavily stained for acetylcholinesterase⁹. In addition, there are many hippocampal afferents from the medial septum; and various lines of evidence²⁰ support the hypothesis that the septohippocampal pathway is cholinergic. Further, it has been demonstrated that electrical stimulation of the septum results in a marked increase in ACh output from both the hippocampus²¹ and cortex²². Therefore, in view of the present findings, activation of the septum by TRH is a possible mechanism mediating the decrease in hippocampal and cortical ACh levels reported by Schmidt in the pentobarbital-pretreated rat.

Thus the septum is highly sensitive to TRH when compared with other brain regions, but with systemic administration it is likely that TRH acts in part through less sensitive brain sites.

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Corticotrophin releasing factor may be modulated vasopressin

CORTICOTROPHIN RELEASING FACTOR (CRF) was the first of the postulated hypothalamic factors controlling the release of hormones from the pituitary gland to be demonstrated biologically¹. However, it is extraordinary that after 24 years of research, techniques which have established the identity of the hypothalamic peptides involved in the release of thyrotrophin, the gonadotrophins and growth hormone, have been unsuccessful when applied to the search for a factor responsible for

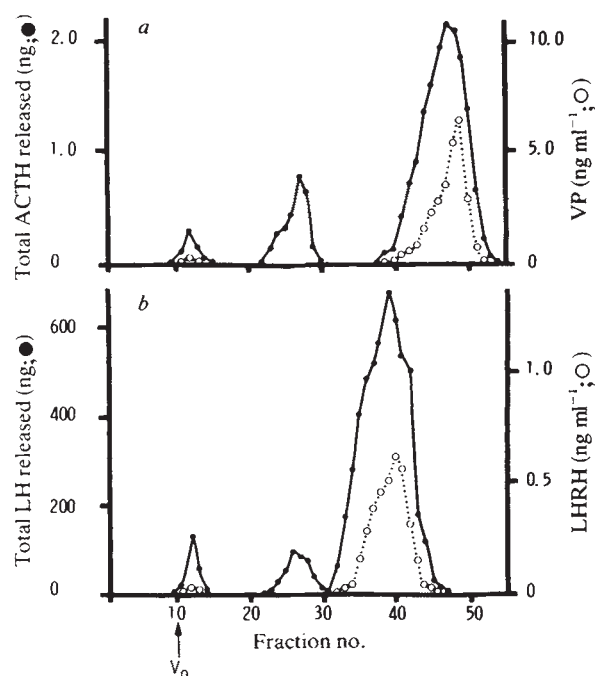


Fig. 1 Chromatography of rat SME on BioGel P2 (200–400 mesh). Twenty rat stalk-median eminences (4 mg tissues wet weight per SME) were homogenised in 1.5 ml 10 mM HCl. After addition of NaCl (9 mg ml⁻¹) and centrifugation (3,000 g) the supernatant was applied to a column (1.5 × 50 cm) of BioGel P2 (200–400 mesh) equilibrated with 10 mM HCl containing NaCl (9 mg ml⁻¹) and ascorbic acid (1 mg ml⁻¹) at 4 °C. The column was eluted with the same buffer and hourly fractions (2.5 ml) were collected. Each fraction was neutralised with 25 µl 1.1 M NaHCO₃ and was diluted 1:5 in the appropriate assay buffer. *a*, ●, CRF bioactivity, as measured by output of immunoreactive ACTH from the cell column; ○, AVP immunoactivity (ng ml⁻¹ in 1:5 dilution). *b*, ●, LHRH bioactivity as measured by output of immunoreactive LH from the cell column; ○, LHRH immunoactivity (ng ml⁻¹ in 1:5 dilution). No significant amounts of ACTH or LH were detected in any of the fractions. V₀, void volume of column.

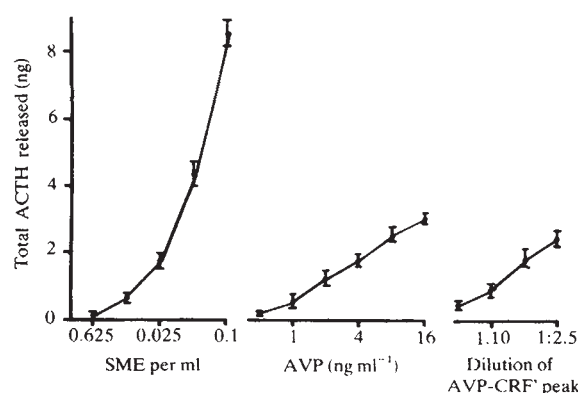


Fig. 2 Comparison of the CRF bioactivity of crude SME (one SME per ml: 1 ml contains neutralised acid extract prepared from the rat stalk-median eminence), synthetic AVP, and the 'AVP-CRF' peak shown in Fig. 1*a* (pooled fractions 43–51). These results were obtained from a chromatogram run in 10 mM HCl containing NaCl (9 mg ml⁻¹) without ascorbic acid. Doses were given randomly in triplicate and results represent mean ± s.e.m.

adrenocorticotrophin (ACTH) release^{2,3}. Vasopressin invariably releases ACTH from the anterior pituitary, but its partial agonistic properties *in vitro*^{4–6}, its low potency *in vivo*⁷ and the isolation of active CRF fractions with little or no vasopressin activity^{2,3,8,9} have led to its rejection as a physiological CRF^{10,11}. However, recent immunological and immunocytochemical findings have led to renewed interest in the role of vasopressin in ACTH release. High concentrations of immunoreactive vasopressin have been found in portal blood and a direct vasopressin-neurophysin containing axonal pathway to the hypophyseal portal system, which seems to be under the influence of the adrenal cortex, has been identified^{12,13}. Using a sensitive releasing factor bioassay system, the perfused isolated anterior pituitary cell column¹⁴, and various immunological techniques, we have presented evidence to support the idea that vasopressin plays a major part in control of ACTH release⁶, and we now report the existence of hypothalamic factors with weak, labile CRF activity which potentiate the CRF activity of vasopressin to give it full agonistic properties indistinguishable from crude extracts of rat stalk median eminence (SME). Thus we propose that CRF is vasopressin modulated by other hypothalamic factor(s) released into the hypothalamo-hypophyseal portal system.

We reported previously that the immunological characteristics of the vasopressin-like material in rat SME were identical to synthetic arginine vasopressin (AVP) although the AVP content could never account for more than 30% of the biological activity of the extract⁶. The luteinising hormone releasing hormone (LHRH) bioactivity of SME, however, was completely in accord with its immunoreactive LHRH content. Surprisingly, when we attempted to remove the 'interfering' AVP from SME by preincubation with AVP antisera, the CRF activity was also specifically and totally quenched⁶.

The CRF biological activity of a crude extract of SME was fractionated by gel filtration (Fig. 1). The first small peak at the void volume (V₀) contained negligible CRF activity (Fig. 1*a*). The material in the second peak (fractions 22–28) appeared to be nonspecific (N_s) in the bioassay as it also released luteinising hormone (LH) (Fig. 1*b*). The major peak of CRF activity (fractions 39–53) eluted in the expected position for AVP and contained the peak of AVP immunoactivity (Fig. 1*a*). The major peak of LHRH bioactivity coincided with the immunoreactive peak of LHRH (Fig. 1*b*). The log-dose response characteristics of the material in the 'AVP-CRF' peak (fractions 43–51) were indistinguishable from those of synthetic AVP, each was a partial agonist in the CRF bioassay when compared with SME (Fig. 2). Using three vasopressin antisera of differing specificities⁶, the vasopressin immunoactivity of this peak was characterised as AVP.

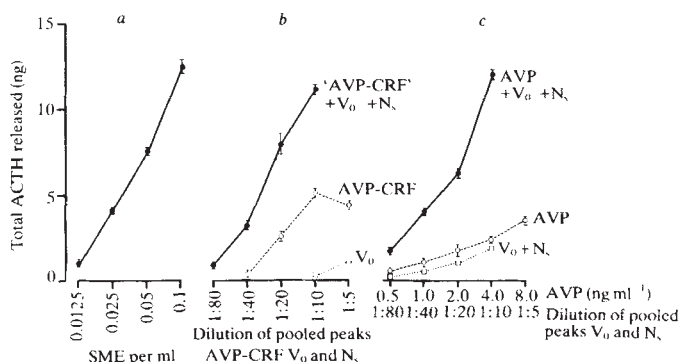


Fig. 3 The synergistic effect of recombination of the CRF peaks in Fig. 1 and synthetic vasopressin. The results were obtained from a chromatogram run in 10 mM HCl containing NaCl (9 mg ml⁻¹) and ascorbic acid (1 mg ml⁻¹) at 4 °C. V₀, N_s and 'AVP-CRF' are pooled fractions 11–31, 22–28 and 43–51, respectively, in Fig. 1.

Thus the material of the major CRF peak resembles synthetic vasopressin immunologically, chromatographically (on BioGel P2) and biologically (in our CRF bioassay) and the lack of full agonist bioactivity of 'AVP-CRF' suggests that chromatography has removed some synergistic factors.

The reappearance of biological activity after recombination of all the fractions after gel filtration of SME has been reported previously¹⁵, but in a recent review, Saffran and Schally³ reported that this work could not be repeated. Initially we failed to observe any potentiation when AVP was recombined with fractions from other areas of the chromatogram. However, when the chromatographic column was equilibrated and developed with eluant containing the antioxidant ascorbic acid, we consistently observed potentiation of the CRF bioactivity after recombination of the 'AVP-CRF' peak with other areas of the chromatogram. This potentiation was also observed with synthetic AVP. Figure 3b shows that the combined 'AVP-CRF', V₀ and N_s fractions had bioassay log dose-response characteristics similar to those of crude SME. Equivalent quantities of synthetic AVP added to a mixture of V₀ and N_s also gave a curve similar to that for SME (Fig. 3c). There was some slight synergism between V₀ and N_s but this activity was unstable after storage for short periods at -20 °C. As crude extracts of normal SME are stable to storage and heat treatment⁶, it seems that AVP has a stabilising influence on the CRF activity of SME.

Studies with Brattleboro rats¹⁵, which are vasopressin deficient, have shown significantly reduced CRF bioactivity in their SME (20% of normal) which is unstable to storage at -20 °C, thus resembling the activity of V₀ and N_s peaks of normal SME. Chromatography of Brattleboro SME yielded V₀ and N_s peaks similar to those shown in Fig. 1 but the 'AVP-CRF' peak, with respect to both CRF bioactivity and AVP immunoactivity, was absent. The Brattleboro LHRH chromatogram was identical in all aspects to that for normal SME¹⁶.

We conclude that the CRF bioactivity of a crude stalk median eminence extract is due to a multi-factor system. Vasopressin is the major component whose potency is modulated by synergistic factors which have weak inherent CRF bioactivity. They lose most of their bioactivity and all of their ability to potentiate vasopressin when separated from it under non-reducing conditions as indicated by the negligible synergism of the components of the chromatogram when ascorbic acid is omitted. In view of the apparent compound nature of CRF bioactivity and the instability of some of its components, it is not surprising that the search for a pure peptide, distinct from vasopressin, with potent CRF activity has been unsuccessful.

This multi factor hypothesis is an attractive one because of the variety of stress situations (physiological, pathogenic, psychological), in addition to circadian rhythm, in which ACTH is secreted. Thus, the weak agonistic properties of vasopressin could be modulated by a specific factor or permutation of factors

in different situations. The factors, in turn, could be involved in the simultaneous release of growth hormone and/or prolactin, which are also released during stress.

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Effect of electroconvulsive shock on monoaminergic receptor binding sites in rat brain

CHRONIC administration of either tricyclic antidepressant drugs or monoamine oxidase inhibitors, which are effective in the treatment of endogenous depression, has been shown to decrease the sensitivity of noradrenaline (NA)-stimulated adenylate cyclase^{1–5} and to decrease the apparent density of β -adrenergic receptor binding sites in rat brain^{6–10}. This alteration in receptor mechanisms by antidepressant drugs seems to be selective, as neither α -adrenergic nor serotonergic receptor binding in rat cortex is altered by the tricyclic drug desipramine⁷. It is therefore of interest to know whether other forms of antidepressant intervention alter receptor mechanisms in the brain and if so, whether they display a time course and selectivity similar to that of antidepressant drugs. Here, we have investigated the effects of a single and of multiple electroconvulsive shocks (ECS) on rat brain monoaminergic receptor binding sites and report that the density of β -adrenergic receptors is decreased.

Electroconvulsive shock (150 mA, 200 ms) was administered to male Sprague-Dawley rats through saline-moistened corneal electrodes. Controls were handled in the same manner except that no current was passed. Shocks were administered for 1 day and for 7 consecutive days. All shocked rats experienced generalised tonic-clonic seizures. Rats were decapitated 24 h after their last shock and the cortex and corpus striatum were dissected from the brains, frozen on dry ice and stored at -85 °C until assayed. The kinetic properties of α -adrenergic, β -adrenergic and serotonergic receptor binding sites were measured in the cortex and those of dopaminergic binding sites were measured in the striatum. The cortices were suspended in 19 volumes of ice-cold 50 mM Tris-HCl buffer (pH 7.7 at 23 °C) containing 0.1% ascorbic acid. The striata were suspended in 39 volumes of ice-cold buffer containing 50 mM Tris-HCl, 2 mM CaCl₂, 5 mM KCl, 120 mM NaCl, 1 mM MgCl₂, 0.1% ascorbic acid and 10 μ M pargyline (pH 7.7 at 25 °C). The tissues were disrupted using a Brinkmann polytron (setting 6, 30 s), and the homogenates centrifuged at 48,000g for 10 min. The pellets were rehomogenised and centrifuged as before. The cortical pellets were resuspended in 19 volumes of buffer and the striatal pellets in 39 volumes of buffer (based on original weight).

Aliquots of tissue equivalent to 10 mg of cortex and to 5 mg of striatum were assayed.

Binding of ^3H -*l*-dihydroalprenolol (^3H -DHA) to β -adrenergic receptors was carried out at 23 °C for 15 min in 0.5 ml 50 mM Tris-HCl buffer (pH 8.1 at 23 °C) containing 0.1% ascorbic acid^{11,12}. Assays were carried out in sextuplicate with half the tubes containing 20 μM propranolol to assess nonspecific binding. The α -adrenergic receptor binding sites were assayed using ^3H -dihydroergocryptine (^3H -DHE) and ^3H -WB-4101 as the binding ligands¹³⁻¹⁶. Dihydroergocryptine is a mixed agonist-antagonist at α -adrenergic receptors and is thought to bind to both an agonist and an antagonist α -adrenergic receptor site in our assay conditions^{13,14}. The antagonist WB-4101 seems to bind only to the α -adrenergic antagonist site¹⁶. Incubations were carried out in sextuplicate at 25 °C for 45 min with ^3H -DHE and for 15 min with ^3H -WB-4101 in 2 ml 50 mM Tris-HCl buffer (pH 7.7 at 25 °C) containing 0.1% ascorbic acid. Nonspecific binding was determined by adding 100 μM *l*-NA to half the tubes. The serotonergic binding site was assayed using ^3H -serotonin (^3H -5-HT) as the binding ligand¹⁷. Tissues were preincubated for 10 min at 37 °C in 2 ml 50 mM Tris-HCl buffer (pH 7.1 at 37 °C) containing 0.1% ascorbic acid, 4 mM CaCl_2 and 10 μM pargyline, an inhibitor of monoamine oxidase. After preincubation, the tubes were returned to ice and ^3H -5-HT was added in sextuplicate. Half the tubes also contained 20 μM nonradioactive 5-HT to assess nonspecific binding. Incubations were carried out for 10 min at 37 °C. The dopaminergic binding site in the striatum was assayed in quadruplicate, using ^3H -spiroperidol (^3H -spiro) as the binding ligand^{18,19}. Tissues were preincubated for 5 min at 37 °C and returned to ice. To assess nonspecific binding, 10 μM (+)butaclamol was added to half the tubes. Incubations were carried out for 20 min at 37 °C in 0.5 ml of the same buffer used to homogenise the tissue. All incubations were terminated by adding 4 ml of ice-cold buffer to each tube and rapidly filtering the samples under reduced pressure through Whatman GF/B glass fibre filters. The tubes and filters were then rapidly rinsed four times with 4-ml aliquots of cold buffer. Filters were transferred to counting vials containing scintillation fluid and counted in a Searle mark III scintillation counter at an efficiency of

Table 1 Maximum specific binding and dissociation constants of monoamine receptor ligands in brain fractions from control and ECS-treated rats

Region	Treatment	Maximum binding (pmol per g tissue)	Dissociation constant (nM)
Cortex	^3H -DHA Control	10.9 $\pm$ 0.9	2.7 $\pm$ 0.5
	^3H -DHA ECS	7.8 $\pm$ 0.6*	2.1 $\pm$ 0.2
	^3H -DHE Control	28.1 $\pm$ 1.6	1.9 $\pm$ 0.3
	^3H -DHE ECS	27.0 $\pm$ 0.7	1.9 $\pm$ 0.1
	^3H -WB-4101 Control	7.8 $\pm$ 0.5	0.28 $\pm$ 0.01
	^3H -WB-4101 ECS	7.9 $\pm$ 0.4	0.24 $\pm$ 0.02
Corpus striatum	^3H -5-HT Control	15.8 $\pm$ 2.0	8.5 $\pm$ 1.4
	^3H -5-HT ECS	14.7 $\pm$ 1.4	7.1 $\pm$ 1.1
	^3H -Spiro Control	24.0 $\pm$ 0.7	0.33 $\pm$ 0.07
	^3H -Spiro ECS	23.3 $\pm$ 0.7	0.37 $\pm$ 0.08

Maximum specific binding and dissociation constants were determined from least-squares fit to Scatchard plots of the data. The results are the mean $\pm$ s.e.m. from 5–8 rats. Statistical analyses were carried out by grouped Student's *t*-test.

* $P < 0.02$.

40–42%. In these conditions, specific binding, defined as the difference between counts bound to tissue in the presence and absence of saturating concentrations of nonradioactive ligand, was between 60 and 90% depending on the assay and the concentration of the ^3H -ligand.

There was no apparent alteration in the α -adrenergic, β -adrenergic or serotonergic receptor binding characteristics after a single ECS. However, Scatchard analysis²⁰ of ^3H -DHA binding to cortical membranes indicated that 7 days of ECS reduced the number of binding sites by approximately 25% compared with the handled controls (Fig. 1). There was no change in the apparent affinity of the binding sites for ^3H -DHA. In contrast to the changes observed in β -adrenergic binding, 7 days of ECS produced no apparent alterations of α -adrenergic or serotonergic binding in the cortex nor of dopaminergic binding in the corpus striatum (Table 1).

As the animals were not anaesthetised, it is possible that a nonspecific stress response contributed to the reduction in the β -adrenergic receptor seen following chronic ECS. To ascertain whether another form of stressful situation would decrease ^3H -DHA binding, we investigated the effects of electric foot shock on β -adrenergic binding. Foot shock was applied by placing the rat in a 28 $\times$ 20 $\times$ 15 cm test chamber containing a grid floor connected to a constant current shock source with a shock scrambler (BRS Foringer shock generator scrambler model SGS-001). The rats received foot shocks of 2 mA intensity and 1.0 s duration at a variable time (VT) schedule with a mean interval of 15 s. The rats were shocked daily for 10 min for 7 consecutive days. Control rats were treated similarly except that no current was delivered to the chamber grid. Rats were decapitated 24 h after their last session in the test chamber. Cortical β -adrenergic receptor binding of 0.7 nM ^3H -DHA in tissue from control ($n = 6$) and foot shock-treated ($n = 6$) rats was found to be 1.76 $\pm$ 0.18 and 1.64 $\pm$ 0.19 pmol per g tissue, respectively, and 7.28 $\pm$ 0.26 and 7.13 $\pm$ 0.23 pmol per g using 4.5 nM ^3H -DHA. This absence of reduction in specific ^3H -DHA binding with foot shock suggests that nonspecific stress alone was not a major factor in reducing the density of β -adrenergic receptors in the brains of rats subjected to chronic ECS.

Electroconvulsive shock has been shown to affect several biochemical parameters of the central noradrenergic system. It increases the activity of tyrosine hydroxylase,²¹ endogenous NA concentration²² and turnover^{22,23}, and the release of NA²², and decreases the high affinity uptake of NA into synaptosomes²⁴. Through these effects, ECS might be expected to increase the availability of NA at the synapse²⁵ and could initiate a neurochemical adaptation at the receptor level. Sulser *et al.* have

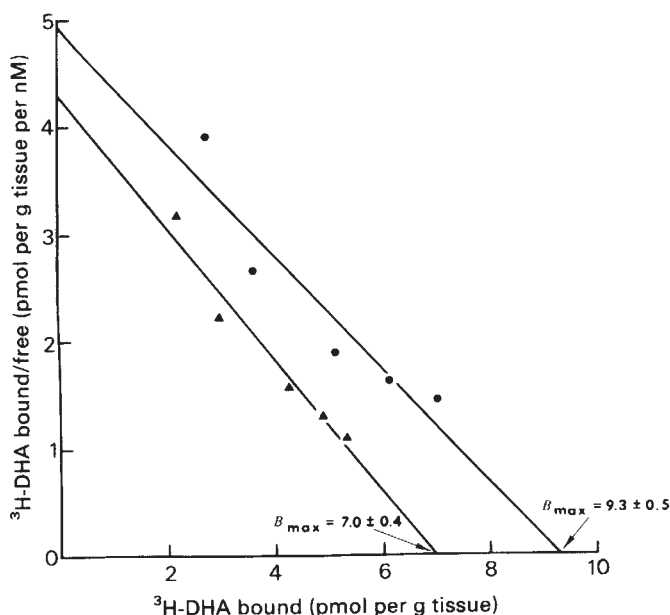


Fig. 1 Scatchard plot of specific ^3H -*l*-dihydroalprenolol binding to cortical fractions from control (●) and ECS-treated (▲) rats. The concentrations of ^3H -DHA used varied from 0.5 to 5.0 nM. This experiment is representative of results obtained from three separate experiments. Data points are the means from 2 control and 2 ECS-treated rats, each assayed in triplicate. Slopes and intercepts of the lines were determined by the method of least squares.

reported that administration of ECS to rats for 8 days reduces the activity of the NA-stimulated cyclic AMP-generating system in limbic forebrain^{2,3}.

The development of neurotransmitter receptor subsensitivity following exposure to agonist compounds or drugs that potentiate the action of existing transmitters has been reported previously^{6-10,26-30}. We report here another example of the development of receptor subsensitivity, the decreased density of β -adrenergic receptors in rat brain cortex following chronic ECS. It is particularly interesting that chronic ECS results in a decrease in cortical β -adrenergic receptor density similar in magnitude and selectivity to that following chronic administration of desipramine⁷. In both cases the time course of reduction of β -adrenergic receptor density closely parallels the reported decrease in activity of the NA-stimulated cyclic AMP-generating system^{2,3}.

These results indicate that the effects on neurotransmission at the β -adrenergic synapse produced by chronic administration of tricyclic antidepressant drugs or ECS are opposite to the effects produced by acute administration of these treatments. Thus, although both tricyclic antidepressants and ECS increase the amount of NA available to interact with receptors in the synapse, the reduction in the number of β -adrenergic receptors after chronic treatment would be expected to result in a decrease in the transmitted message. The reduction in activity of the NA-stimulated cyclic AMP-generating system following chronic administration of either tricyclic antidepressants or ECS¹⁻⁴ seems to support this.

The selectivity of the response of neurotransmitter receptors to chronic ECS reported here agrees closely with that reported for desipramine⁷ and suggests that an adaptation of central β -adrenergic receptors may be important for the therapeutic effects of antidepressant treatment.

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Bio-selective membrane electrode using tissue slices

MEMBRANE electrodes based on living bacterial cells¹ as biocatalysts have been shown to be useful sensors for amino acids and other biomolecules. We report here that we have now developed a highly effective membrane electrode for glutamine using slices of porcine kidney tissue as the biocatalytic layer. This tissue-based electrode yields excellent selectivity and sensitivity while having the additional advantages of simplicity and low cost. Further, the tissue-based electrode shows a much longer useful lifetime than comparable electrodes prepared with the isolated porcine enzyme.

The electrode assembly (Fig. 1) was prepared by immobilising a thin (~0.05 mm) slice of porcine kidney at the surface of an ammonia gas sensing membrane electrode (Orion model 95-10) by means of a dialysis membrane or a mesh of monofilament nylon. Pork kidneys were obtained from freshly killed animals and stored at 4 °C until use. In separate experiments the kidney slices showed high glutaminase activity as indicated by their ability to metabolise glutamine rapidly to products including ammonia. Moreover, the enzyme activity was retained within the intact tissue over extended time periods. This finding is consistent with the view that the glutaminase enzyme is situated in the kidney cell mitochondria².

Evaluations of the potentiometric response of the kidney slice electrode were made at 26 °C in 0.1 M phosphate buffer, pH 7.8, containing 0.02% sodium azide as preservative. Between experiments, the electrodes were stored in the buffer at room temperature. Figure 2 shows a typical calibration curve of the electrode for L-glutamine. The slope, linear range and detection

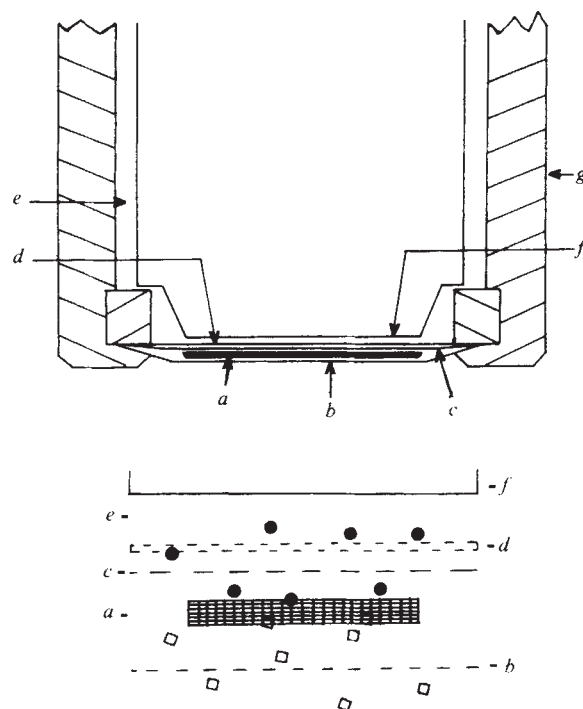


Fig. 1. Top, schematic diagram of the kidney tissue-based membrane electrode for glutamine; a, the slice of porcine kidney tissue; b, a monofilament nylon mesh; c, the protective dialysis membrane; d, the gas-permeable membrane; e, the internal electrolyte solution; f, the pH sensing glass membrane; and g, the plastic electrode body. (Components d-g represent the Orion model 95-10 ammonia gas sensing electrode.) Bottom, expanded view of the electrode sensing tip showing components and phases. □, Glutamine; ●, ammonia; identifying letters as above.

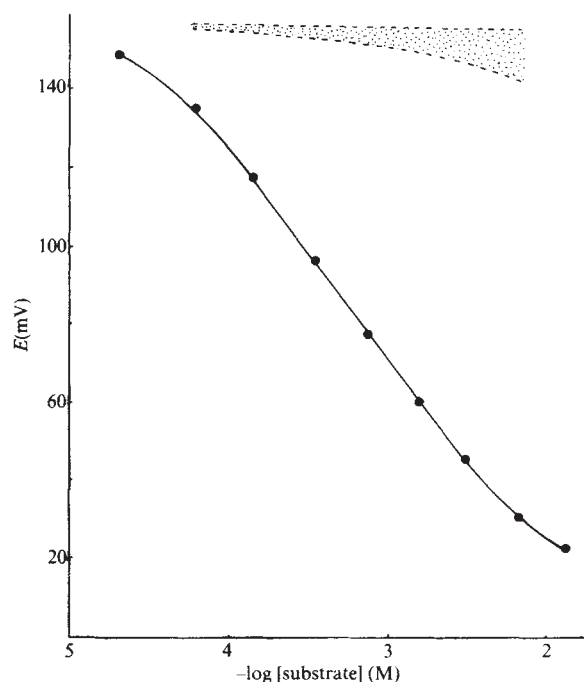


Fig. 2. Potentiometric response and selectivity of the kidney-based electrode in 0.1 M phosphate buffer, pH 7.8, with 0.02% sodium azide preservative at 26 °C. Solid line shows the electrode response as a function of glutamine concentration over the range 2×10^{-2} to 5×10^{-5} M. Dotted region shows the maximum response to the 13 possible interference compounds tested.

limit remained unchanged over at least 28 d of electrode use. Typically, the electrode attained a steady potential value within 5–7 min of each addition of glutamine to the test solution.

The kidney tissue electrode yielded negligible response to compounds causing possible interference such as urea, L-alanine, L-arginine, L-histidine, L-valine, L-serine, L-glutamic acid, L-asparagine, L-aspartic acid, D-alanine, D-aspartic acid, glycine or creatinine. This high degree of potentiometric selectivity compares favourably with that of conventional electrodes³.

Parallel electrode experiments were also carried out using the isolated porcine kidney glutaminase enzyme (Sigma, grade Vi), known to be thermally unstable⁴. Small volumes (~15 µl) of enzyme suspension were immobilised at the surface of the ammonia gas sensing electrode by means of a dialysis membrane, and electrode response to L-glutamine was evaluated in the buffer medium. Freshly prepared enzyme electrodes yielded moderate potentiometric response (44 mV per 10-fold change in substrate concentration) but lost more than 50% of their activity within 2 d and showed negligible response after 4 d at room temperature. Thus, it is clear that the kidney tissue electrode is a very substantial improvement, in terms of lifetime and response, over the conventional enzyme electrode. In view of continuing advances³ in the development of potentiometric membrane electrodes, other tissue-based electrodes of fundamental and practical significance can be expected.

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Elevation of protein synthesis is a complex response to fertilisation

PROTEIN SYNTHESIS in unfertilised eggs of the sea urchin proceeds at a low rate. However, fertilisation triggers a series of reactions beginning with changes in intracellular cation concentrations within a few seconds of sperm entry and extending to a cascade of more complex events¹. As these events are sequential in time, each might depend on the preceding event, or there might be a parallel series of independent pathways. The latter hypothesis was shown to be correct by Epel *et al.*², who demonstrated that artificial activation with ammonia failed to initiate events such as sodium influx and the cortical reactions but did initiate a variety of others including potassium conductance, protein synthesis, DNA synthesis and mRNA polyadenylation.

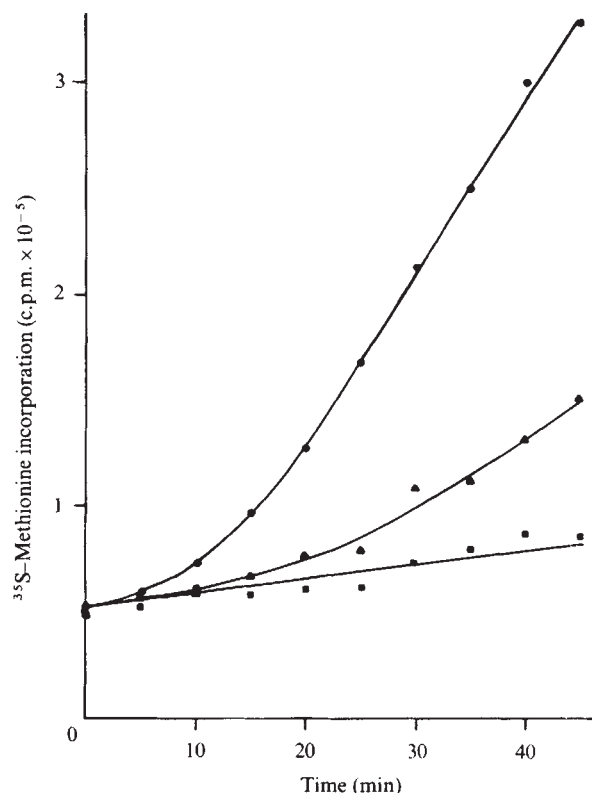


Fig. 1 Elevation of protein synthesis as a function of activation method. Eggs (2×10^5 per ml, total volume 35 ml) were pulsed with $10 \mu\text{Ci ml}^{-1}$ ^{35}S -methionine ($1,030 \text{ Ci mmol}^{-1}$) for 60 min at 17 °C. The sea water contained 50 mg ml^{-1} each of penicillin and streptomycin adjusted to pH 8.1 with NaOH. After incubation, the eggs were washed 3 times by pelleting them at 200 g. The final pellet was resuspended in 15 ml and distributed equally to each of 3 culture vessels at 6.7×10^4 eggs ml^{-1} (final volume 35 ml). At time zero, 2.0 ml of sea water was added to one of the vessels (■), 2.0 ml 200 mM NH_4Cl was added to the second culture (▲) and 2.0 ml of concentrated sperm suspension (1 drop sperm as obtained from the gonads in 5 ml sea water) was quickly squirted into the third culture (●). Taking precautions to avoid cross-contamination, 1.0-ml samples were removed from each culture at 5 min intervals. The samples were squirted directly into an equal volume of ice-cold 20% trichloroacetic acid (TCA) plus 50 mM methionine. The eggs and embryos were pelleted, washed once with the TCA mixture, and heated to 90 °C for 30 min. During heating each tube was vigorously agitated several times. Precipitates were collected on Whatman glass fibre filters (GF/C), washed 4 times with 2 ml 10% TCA plus 25 mM methionine, and once with 95% ethanol, and the disks were then placed in scintillation vials. Protein was digested off the disks in sealed vials by adding 0.5 ml 0.2 M NaOH and heating to 60 °C for 2 h. The hydrolysates were cooled, neutralised with 1 M acetic acid, dissolved in 10 ml Instagel scintillation fluid, and counted.

We have been studying the elevation of protein synthesis in detail. The rate of protein synthesis increases severalfold during the early cleavage stages³ and eventually reaches an absolute rate which is 100 times higher than that in the mature oocyte⁴. Kaumeyer *et al.*⁵ and Jenkins *et al.*⁶ have demonstrated that unfertilised eggs contain 'masked' messenger ribonucleoprotein particles which are not translatable *in vitro* unless modified or deproteinised. Most of the increase in the rate of protein synthesis following fertilisation is due to the recruitment of this oogenetic mRNA as a result of unmasking⁷. However, a significant portion of the rise also results from an increase in translational efficiency⁸. As defined by Palmiter⁹, translational efficiency is the number of completed polypeptides released per unit time per message. The change in efficiency following fertilisation of sea urchin eggs is reflected in an increase in the rate of ribosome movement (that is, the time required to complete an average size protein decreases after fertilisation) coupled to a concomitant increase in initiation rate. The net effect on protein synthesis of an increase in number of mRNAs available for translation and an increase in translational efficiency is multiplicative. We present evidence here that these two processes are under separate controls.

As Fig. 1 shows, in *Strongylocentrotus purpuratus* eggs the rise in protein synthesis induced by ammonia lags behind the rise caused by fertilisation. The same response was observed in a different species by Epel *et al.*². They found that even though the

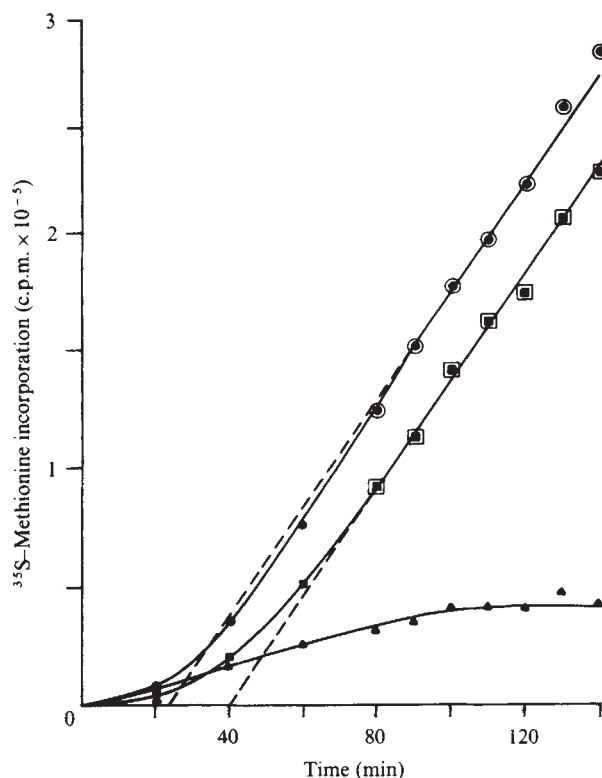


Fig. 2 Determination of ribosome transit time for ammonia-activated, unfertilised eggs. A culture of eggs (32,000 per ml) in sea water containing penicillin and streptomycin (pH adjusted to 8.1 with NaOH) was treated with sufficient 200 mM NH_4Cl to bring the final concentration of NH_4^+ to 10 mM. Five minutes later, ^{35}S -methionine was added ($5 \mu\text{Ci ml}^{-1}$; 900 Ci mmol⁻¹) plus unlabelled methionine to yield a final exogenous amino acid concentration equal to $0.32 \mu\text{g ml}^{-1}$. The addition of isotope was defined as time zero, after which 5.0-ml samples were removed and processed as described elsewhere⁸. Transit times were obtained by multiplying by two the distance between the extrapolated 'total' and 'soluble' lines. All extrapolations were based on those points after the polysome phase had reached steady-state labelling, when the flow of radioactivity into total and soluble proteins is linear⁸. Points used for extrapolation are outlined. ●, Total incorporation; ■, incorporation into soluble proteins; ▲, incorporation into polysomes.

Table 1 Ribosome transit times for ammonia-activated eggs

Experiment	Isotope	Transit time (min)
UF eggs	^3H -Leu	41.6
UF eggs	^{35}S -Met	38.6
		Average = $40.4 \pm 4.0^\dagger$
30 min zygotes	^3H -Leu	14.0
30 min zygotes	^{35}S -Met	19.0
		Average = $18.0 \pm 4.4^\ddagger$
NH_3 -activated eggs	^3H -Leu*	33.2
NH_3 -activated eggs	^{35}S -Met*	39.1
		Average = $36.2^\ddagger$

Cultures of unfertilised (UF) eggs and zygotes were handled as described elsewhere⁸. In activation experiments, unfertilised eggs in Millipore-filtered sea water containing $50 \mu\text{g ml}^{-1}$ each of penicillin and streptomycin (pH adjusted to 8.1) were treated with sufficient 200 mM NH_4Cl to bring the final concentration of ammonium ion to 10 mM. Five minutes later, either ^3H -leucine (New England Nuclear, $57.4 \text{ Ci mmol}^{-1}$) or ^{35}S -methionine (Amersham, 900 Ci mmol^{-1}) was added to the culture. The addition of isotope was defined as time zero after which samples were removed and processed as described in Fig. 2.

* The total final exogenous amino acid concentrations, including isotope, in $\mu\text{g ml}^{-1}$ were 0.32 for methionine ($32,000 \text{ eggs ml}^{-1}$) and 1.45 for leucine ($23,000 \text{ eggs ml}^{-1}$). The final exogenous radioactivity was $5 \mu\text{Ci ml}^{-1}$ in each experiment.

† The averages of 5 determinations for each stage of development ± 1 s.d. The 2.2-fold difference in means yields a $t = 7.48$ for 8 degrees of freedom or $p < 0.001$, indicating that the difference is significant at the 99% confidence level in a standard t test⁸.

‡ The average transit time for ammonia-activated eggs is within 1 s.d. of the unfertilised egg. A z test¹² for group variance indicates that this value is probably a subset of the parent group unfertilised egg value, but not of the parent group zygote value.

rise in protein synthesis induced by ammonia lagged behind the rise caused by fertilisation, the same level of recruitment of ribosomes (and thus of mRNA) into polysomes was attained in both ammonia-activated and fertilised eggs. These observations suggested to us that ammonia activation may cause unmasking of mRNA but not stimulate a change in translational efficiency.

To test the hypothesis that ammonia-activated eggs recruit mRNA but fail to increase the rate of ribosome movement, we measured the average transit time in ammonia-activated eggs using the method developed for time-course incorporation into sea urchin eggs and embryos as shown in Fig. 2. The results are summarised in Table 1. We found that ammonia-activated eggs require as much time as unfertilised eggs to complete an average size polypeptide. This is about twice as long as the time required by zygotes. Thus, the increase in protein synthesis caused by ammonia treatment is the result only of an increase in the amount of available mRNA in the absence of an increase in the rate of ribosome movement. Therefore, the two events responsible for the rise in protein synthesis following fertilisation of the *S. purpuratus* egg must be independent of one another.

The normal acceleration of synthetic events following fertilisation seem to result from a transient elevation in intracellular pH caused by a massive influx of sodium ions coupled to a loss of protons¹⁰. Ammonia is believed to function as an activating agent by raising the internal pH of the egg. Weak organic bases enter the unfertilised egg in the uncharged form and then gain protons, thus raising the intracellular pH and mimicking the normal $\text{Na}^+ - \text{H}^+$ exchange¹¹. This rise in internal pH must stimulate unmasking, but as ammonia does not stimulate a change in the rate of ribosome movement, the increase in translational efficiency in zygotes must depend on an as yet unidentified mechanism.

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Efficiency of protein synthesis after fertilisation of sea urchin eggs

ELUCIDATION of the mechanisms involved in the cytoplasmic control of protein synthesis is essential for an understanding of the initiation of embryogenesis. On fertilisation, echinoid eggs increase their rate of protein synthesis many-fold, independent of any increase in ribosomal or mRNA synthesis¹⁻⁴. Within 2 h of fertilisation the number of ribosomes in polyribosomes increases about 25-fold without any change in the size distribution of poly ribosomes^{5,6}. In addition, the polyribosome number doubles again by gastrulation. However, the total rate of protein synthesis increases 113-fold from egg to gastrulation⁷. The discrepancy between the number of ribosomes recruited into polyribosomes, an increase of ≈ 50 fold, and the total increase in the rate of protein synthesis, a 113-fold increase, could be explained if there were also an increase in the rate of movement of ribosomes along each message (elongation) and/or an increase in the rate of release of completed peptides (termination). We report here that the translational rate is increased about 2.5-fold after fertilisation in the sea urchin, *Strongylocentrotus purpuratus*. The average transit time (elongation plus termination time for an average-sized peptide) was measured for these eggs and several stages of embryos.

The transit times of eggs and embryos of *S. purpuratus* were measured at 12°C by modifications of Fan and Penman's time course method^{8,9}. The low temperature used is that of the sea urchin's normal habitat and gives accurately measurable, slow elongation rates. The time course method is independent of variations in the rate of initiation of peptide synthesis, the number of polyribosomes and the specific activity of the amino acid pools. The method compares the ³H-amino acid incorporation into total peptides (incomplete nascent peptides still covalently bound to tRNA on the polyribosomes plus completed, released peptides) and the incorporation into released peptides determined after removal of the polyribosomes by centrifugation. One transit time after the amino acid pools have equilibrated, the rates of incorporation into total peptides and released peptides should be equal and graphically form two parallel lines separated by one-half the transit time⁸. In our experiments these lines, shown in Fig. 1 as open circles and solid circles respectively, did not become parallel but diverged with time. Presumably, this occurs because newly synthesised histones and other basic proteins attach to large deoxyribonucleoprotein or ribonucleoprotein particles¹³⁻¹⁵ and are removed during centrifugation. Analysis of the polyribosomal pellets for peptidyl-tRNA and peptides by DEAE-cellulose chromatography^{9,12} did show the presence of 15-55% released peptides. When the counts in the released peptide fraction

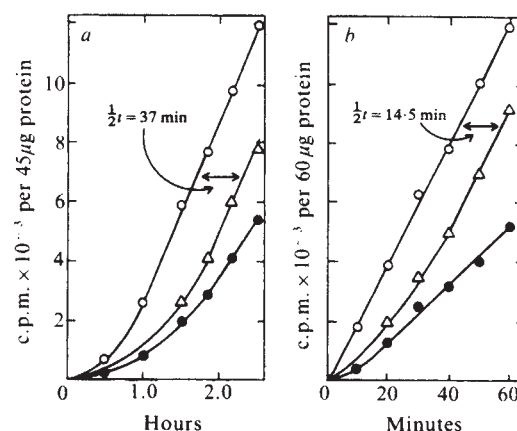


Fig. 1 Representative determinations of the average mRNA transit time in *S. purpuratus* unfertilised eggs (a) and 2-4-cell embryos (b). The time course methods of Fan and Penman⁸ and Palmiter⁹ were modified for sea urchins to determine elongation rates. Eggs and embryos were cultured in rotating tubes at 12°C with ³H-leucine at various specific activities and at various culture densities as specified in Table 1. Samples of 1 ml were removed at the times indicated and added to 40 ml of -2°C buffer I (0.2 M KCl, 0.05 M triethanolamine, 0.01 M MgOAc₂, pH 7.8) to stop synthesis and preserve polyribosome structures. The eggs and embryos were collected by centrifugation at 500g_{max} and washed by an additional 40 ml of buffer I; 1 ml of buffer I containing 2-3 mg ml⁻¹ purified yeast 4S RNA to compete for ribonuclease activity, 0.5% Triton X-100 and 0.5% Na deoxycholate was added to each sample. The eggs or embryos were homogenised either by rapid passage through a no. 20 G needle fitted with 22-μm Nitex Bolting or with a dounce homogeniser. The fertilisation membrane of the embryos had been softened by exposure to 50 mM 3-amino-1,2,4-triazole during fertilisation¹⁰. The homogenates were centrifuged in conditions normally used for removal of mitochondria or larger organelles, that is, at 14,000g_{max} for 10 min at 2°C. The incorporation of ³H-leucine into precipitable peptides was essentially identical in aliquots taken before and after this centrifugation ($\pm 7\%$), indicating complete homogenisation of the eggs and embryos and total solubilisation of membranes, organelles and labelled cellular proteins. Aliquots from the low-speed supernatant, therefore, represented total incorporation (○). To eliminate sampling variations of about 10% the incorporation data were normalised to an average protein concentration after the determination of the total protein content by a modified Lowry procedure¹¹. The low-speed supernatant was centrifuged at 138,000g_{max} for 2 h at 2°C in a Beckman type 65 rotor to remove polyribosomes. Aliquots of the resulting high-speed supernatant were removed to measure completed proteins released from the polyribosomes (●). All aliquots for counting were precipitated in 7.5% (w/w) trichloroacetic acid containing 0.1 mg ml⁻¹ unlabelled L-leucine, and collected on glass fibre disks, digested with N-chlorosuccinamide (Amersham) and counted in a toluene-based scintillant. They were not heated because it is not necessary to hydrolyse the ³H-leucyl-tRNA which occurs in both the aliquots of total incorporation and the released peptides fraction, and because heating selectively removes small peptidyl-tRNAs from the total incorporation. Peptides and peptidyl-tRNA in the S-138 pellet were separated on DEAE-cellulose^{9,12} as described in Fig. 2. Counts from the proportion of the pellet that eluted as peptides were added to those from the high-speed supernatant for total released peptides (Δ). The horizontal distance between the parallel lines for total incorporation (○) and total released peptides (Δ) represent one-half an average transit time, which was 37 and 14.5 min, respectively, for a and b.

recovered from the polyribosomal pellets were added to those of the released peptides in the high-speed supernatant, parallel lines were obtained and transit times determined (Fig. 1, open circles and triangles). The 2-h centrifugation time for polyribosomes was chosen for removal of peptidyl-tRNA with minimal co-centrifugation of released peptides (Fig. 2).

Table 1 summarises the results obtained by time course measurements such as those in Fig. 1. No variation in stage-specific transit times was observed by varying culture conditions such as the concentrations of amino acids or centrifugation times. This showed that, as predicted, the transit times we measured were independent of the specific activities of the amino acid pools, and that our analysis of the released peptides co-sedimenting with polyribosomes was valid and accurate. At 12°C the transit time is about 69 min per average length peptide chain in unfertilised eggs, which is about 2.5-fold longer than for embryos (Table 1). The transit times were 20-24 min for pre-cleavage embryos, 29 min for embryos undergoing 2nd cleavage during the experiment, and 28-32 min for embryos in the 4th cleavage. Therefore, the translational rate increased as early

Table 1 Average mRNA transit time in *S. purpuratus*

Stage	Transit time (min at 12 °C)	μM leucine†	mCi ^3H per μmol leucine	Culture density‡
Unfertilised eggs	66	0.52	24.1	13,800
	64–69*	0.93	13.4	32,100
	72–78	1.36	12.3	21,400
	66–70	2.42	8.3	57,100
Average	69			
Fertilised eggs (60 min, 12 °C)	20–24	199.0	0.10	30,900
Embryos (2–4 cells)	29*	49.8	0.25	13,100
	29	82.9	0.20	28,600
Embryos (16–32 cells)	28–32	199.0	0.10	28,600

The transit times were determined as in Fig. 1 except that samples marked with an asterisk were centrifuged for 2.5 h. The range of graphical error is given where it is greater than 1 min.

† The initial concentration in seawater. Because the rate of amino acid uptake by eggs is very slow compared with embryos¹⁶, the concentration and radiospecific activities of amino acids is necessarily very different.

‡ The number of eggs or embryos per ml was calculated assuming 40 ng protein per egg or embryo¹⁷.

after fertilisation as we were able to obtain accurate transit times, and did not vary significantly through these early stages.

How valid is the measurement of the transit times, which depend on the weight average molecular weight ($\overline{MW}$) of the peptides being synthesised? Bradhorst¹⁸ identified 400 distinctly resolved peptides synthesised before and 30–60 min after fertilisation of *Lytechinus pictus* eggs and found that at least 90% of the spots co-electrophoresed and had similar relative intensities. He found similar results for *S. purpuratus* eggs and zygotes and concluded that there was a quantitative rather than a qualitative regulation of the translation of stored mRNA after fertilisation. Other evidence which supports this conclusion is the similar sedimentation of newly synthesised peptides from eggs and embryos of *L. pictus* in SDS-urea sucrose gradients¹⁹, a similar average $\overline{MW}$ distribution of *S. purpuratus* nascent chains on SDS-polyacrylamide gels²⁰, and a similar size distribution of *S. purpuratus* polyribosomes²⁰. Therefore, the increased translational rate we observed after fertilisation is not due to a decrease in the weight average peptide length.

At first glance, our measured transit times of about 29 min at 12 °C for embryos seem long compared with those of mammalian systems measured at 36 °C and summarised in Table 2. However, Craig^{21,22}, in a very thorough and careful manner, has shown that the transit times increase dramatically (7–50-fold) at low temperatures (Table 2). Mouse L-cells synthesising an average peptide chain length of 85,000 daltons have an average transit time of 22 and 13 min at 10 °C and 15 °C, respectively. Reticulocytes synthesising α and β -globin ($\overline{MW}$ = 17,000) have an average transit time of 20.7 and 6.4 min at 10 °C and 15 °C, respectively. Therefore, the transit times observed for *S. purpuratus* embryos are not unusually long compared with other systems at low temperatures, especially considering tissue variations such as those in Table 2 for hamster ovary cells and chick oviduct at 35–36 °C. However, the transit time of 69 min for unfertilised *S. purpuratus* eggs is unusually long, presumably reflecting the quiescent state of the eggs and their need to conserve materials for later development.

In a paper published after submission of our manuscript, Brandis and Raff²⁰ similarly report long transit times in *S. purpuratus* eggs at 16.5 °C and show a 2–3-fold acceleration following fertilisation. Our observations at 12 °C are in close quantitative agreement if the Q_{10} for elongation is 3 in sea urchins. Changes in elongation rates have also been observed, by Palmiter⁹, in chick oviducts. Stimulation of the oviduct with oestrogen caused a 1.5-fold increase in the elongation rate compared with unstimulated oviduct.

There are several possible reasons for the failure of others to detect the difference between the translational efficiency of eggs and of embryos in sea urchins. For example, Humphreys¹⁹ measured the incorporation of ^{14}C -amino acids into peptides

after only 10 and 20 min of labelling at 18 °C. This was probably too short to achieve equilibrated labelling of the nascent peptides on the polyribosomes, especially for eggs in which he observed an 8-min lag before labelling of the total cellular peptides became linear. Both linear kinetics and complete labelling of nascent peptides are necessary for accurate determinations of transit times. Furthermore, Craig has recently demonstrated the temperature dependence of transit times in mammalian systems^{21,22}; this makes the 1–2-min transit times determined by Humphreys¹⁹ for eggs and embryos at 18 °C seem unreasonably rapid.

Felicetti *et al.*²³ found that, within minutes of fertilisation of *Paracentrotus lividus* eggs, there was a threefold increase in the activity of T1 elongation factor plus a 1.5-fold increase in the activity of T2 elongation factor. The rapid activation of these factors suggests mobilisation of pre-existing proteins. Whether the activity of elongation factors increases only to keep pace with increasing numbers of polyribosomes or whether they are also responsible for increased rates of translation has not been known, but it is now apparent that some of the factors contribute to an increase in translational efficiency.

As the polyribosome size in embryos is the same as that in eggs²⁰, initiation factors must also be available to keep pace with both the increased translational efficiency and the increased recruitment of ribosomes into polyribosomes. Whether these initiation factors pre-exist in an active form, are mobilised to an active form at fertilisation, or are newly synthesised, is not known. However, in the mid-blastula stage the supply of initiation factors does not keep pace with the demand, as about 50% of the mRNAs are not fully loaded with ribosomes²⁴.

What is the relationship of our results to previous studies on the activation of protein synthesis at fertilisation? We propose that the increased rate of elongation after fertilisation will account for observed differences between the increased numbers of ribosomes in polyribosomes^{5,6} and the changes in the absolute rate of protein synthesis⁷. The observed recruitment of ribosomes and mRNA into polyribosomes after fertilisation could occur by a release of mRNAs from inhibited, preformed messenger ribonucleoprotein particles^{4,14} and/or by an increase in the rate with which ribosomes attach to mRNA and initiate protein synthesis^{25–28}. Humphreys⁵ accurately determined the per cent of ribosomes in polyribosomes in *L. pictus* by measuring the ribosomal RNA content of these fractions. He found an average of 0.75% of the ribosomes in polyribosomes in unfertilised eggs and about 20% as polyribosomes in 2–6-h (18 °C)

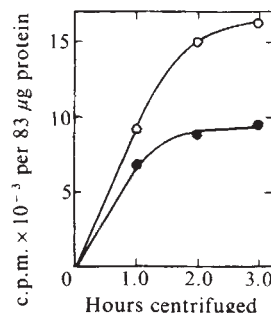


Fig. 2 Determination of optimum centrifugation times for the polyribosome fraction. The low-speed supernatant from eggs labelled with ^3H -leucine was centrifuged at 138,000 g_{max} at 2 °C for 1, 2 and 3 h to pellet polyribosomes. The ribonucleoprotein pellets thus obtained were subjected to DEAE-cellulose chromatography to separate released peptides and peptidyl-tRNA^{9,12}. The polyribosomal pellets were dissolved overnight at 0 °C in 0.5 ml of 3 M LiCl, 6 M ultra pure urea (Schwarz/Mann), 50 mM NaOAc and 5 mM dithiothreitol, pH 5.2, and stored at –80 °C. The solubilised pellet was diluted 30-fold with 8 M ultra pure urea at pH 5.4 containing 6 mM β -mercaptoethanol and applied at room temperature to a 0.5 $\times$ 1 cm DEAE-cellulose column pre-equilibrated with buffer II (0.1 M NaOAc, 8 M ultra pure urea, 6 mM β -mercaptoethanol, pH 5.6). Peptides were eluted with buffer II and peptidyl-tRNA was eluted with buffer III (0.75 M NaOAc, 8 M ultra pure urea, 6 mM β -mercaptoethanol, pH 5.6), and buffer IV (1 M KCl, 0.1 M Tris-HCl, 8 M ultra pure urea, 6 mM β -mercaptoethanol, pH 7.4). The total incorporation associated with the polyribosomal pellet (○) and the fraction occurring as ^3H -peptidyl-tRNA (●) are shown. At 2 h peptidyl-tRNA in the pellet reaches a maximum.

Table 2 Temperature dependence of transit times

Tissues	Transit time (min)			MW*	Ref.
	10 °C	15 °C	35–36 °C		
Mouse L-cells	22.0	13.0	2.0	85,000	21
Reticulocyte cells	20.7	6.4	0.42	17,000	22
Hamster ovary cells			2.0	85,000	8
Chick oviduct			3–4	38,000	9

* Weight average MW.

embryos, a total increase of 27-fold. By measuring the absorption of polyribosomes and monoribosomes at 260 nm, Infante and Nemer⁶ found the percentage of ribosomes as polyribosomes to be 5% in unfertilised eggs, 25% in early cleavage stages, 50% in blastulae and 40% in gastrulae of *S. purpuratus*, a stimulation of 8–10-fold. The largest discrepancy between these data and those for *L. pictus* is for the unfertilised egg. How much of this difference is due to species variation and how much to methods is not known. If, however, we assume that Humphrey's more accurate method establishes the lower limit for polyribosomes in unfertilised eggs at slightly less than 1%, the maximum increase in polyribosomes in *S. purpuratus* by gastrula stage would be 50-fold. In the absence of better data we will use this value for comparison with changes in the total rates of protein synthesis.

Absolute rates of protein synthesis are difficult to obtain because of the 25-fold slower uptake of amino acids by eggs compared with embryos¹⁶ which results in a large difference in the specific activities of their amino acid pools. Regier and Kafatos⁷, however, measured the specific radioactivity of precursor aminoacyl-tRNA pools to obtain accurate data. They found a 113-fold stimulation in the absolute rate of protein synthesis in *S. purpuratus* at the gastrula stage compared with unfertilised eggs. Although this value must theoretically include the 50-fold increase in ribosomes as polyribosomes by the gastrula stage, it is more than twice the latter. As newly synthesised ribosomes are less than 10% of the total ribosome population by gastrulation and as there is also some degradation of old ribosomes²⁹, increased numbers of ribosomes cannot account for any significant increase in rates of protein synthesis. The difference between the 113-fold stimulation in the absolute rate of protein synthesis and the 50-fold increase in polyribosomes can be accounted for by the increased efficiency of translation we report here³⁰.

The unmasking of maternal messenger RNA and/or the increased availability of ribosomal subunits to initiate protein synthesis result in a very large stimulation of protein synthesis after fertilisation, as shown by Humphreys⁵. However, the additional 2.5-fold increase in the rates of elongation can no longer be ignored as contributing to the observed activation of these eggs and the probable doubling of the growth rate of these embryos.

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2-5A synthetase: assay, distribution and variation with growth or hormone status

PROTEIN SYNTHESIS in cell-free systems from interferon-treated cells and rabbit reticulocyte lysates is exceptionally sensitive to inhibition by double-stranded RNA (dsRNA)^{1,2}. Two distinct mechanisms seem to be involved: (1) inactivation of initiation factor eIF2 by dsRNA-mediated phosphorylation and (2) degradation of mRNA by one or more nucleases which are activated by sub-nanomolar levels of an unusual oligonucleotide, pppA2'p5'A2'p5'A (refs 3–10). The enzyme responsible for synthesising pppA2'p5'A2'p5'A requires dsRNA for activity, binds to poly(I) · poly(C)-Sephacrose, and can be used conveniently in this insoluble form to synthesise pppA2'p5'A2'p5'A and related oligomers^{4,11}, collectively referred to as 2-5A. The discovery of relatively high levels of 2-5A synthetase in rabbit reticulocyte lysates¹² raised the possibility that the 2-5A system might be found in other cells which have not been treated with interferon and might, therefore, have a wider significance. We have developed a more convenient assay for the synthetase based on its activity when bound to poly(I) · poly(C)-paper rather than poly(I) · poly(C)-Sephacrose and show here that this enzyme is distributed widely in mammalian cells. Moreover, different cells or tissues have widely differing levels of this enzyme and in the case of chick oviducts there is a substantial increase in synthetase level after withdrawal from stimulation by oestrogen.

The 2-5A synthetase from interferon-treated mouse L cells or rabbit reticulocyte lysates was equally stable and active when bound to poly(I) · poly(C)-paper or poly(I) · poly(C)-Sephacrose. However, the paper has a number of advantages, especially reproducibility of preparation, stability to storage and convenience in use, particularly with multiple small volumes. There is little or no loss of poly(I) · poly(C) from the paper during prolonged incubation (>10 d) with the bound enzyme, allowing the same paper to be re-used several times. The paper-bound enzyme retains full activity on storage for at least 6 weeks at –70 °C.

One square centimetre of poly(I) · poly(C)-paper was at least five times more than enough to bind all the 2-5A synthetase available in a 200-μl extract from reticulocytes or interferon-treated L cells. There was a linear relationship between the amount of extract used in the assay and the amount of 2-5A synthesised. When different amounts of cell sap from interferon-treated cells were mixed with sap from control cells before adsorption of the synthetase to poly(I) · poly(C)-paper, the yield of 2-5A after incubation was proportional to the amount of interferon cell sap used. The levels of ATP used were not limiting. Optimum conditions for synthesis of 2-5A were identical with the chick oviduct, reticulocyte or interferon enzyme bound to poly(I) · poly(C)-paper. Therefore, these conditions

were used throughout the survey of synthetase levels in different cells and tissues described in Table 1.

2-5A synthetase was detected readily in all the mammalian extracts tested, but the amount of enzyme activity varied more than 1,000-fold (Table 1). It was lowest in MRC5 cells in which there was little change in level with passage number, with the possible exception of a significant decrease at passage 55 just before senescence at passage 57. There was slightly more enzyme in pig lymphocytes, with or without stimulation by phytohaemagglutinin. Significantly higher levels were obtained in mouse erythroleukaemia (Friend) and in human lymphoblastoid (Namalva) cells. The fact that the different Friend cell lines F4N, F4N+2 and F4N+ (ref. 13), have different levels is

Table 1 2-5A synthetase levels in a variety of cells and tissues

	Source	Specific activity (pmol 2-5A per A ₂₆₀ per h)
1	Interferon-treated mouse L cells (spinner culture)	600
	Control cells	1
2	Interferon-treated human diploid fibroblasts (ICRF23)	600
	Control cells	10
3	Rabbit reticulocytes	300*
4	Chick oviduct (see Table 2)	5–200
5	†Guinea pig mammary gland:	
	pregnant	70
	lactating	5–50
	2 day-withdrawn	60–80
	4 day-withdrawn	100
6	†Mouse erythroleukaemia (Friend) cells:	
	F4N	10
	F4N+	4
	F4N+2	115
7	†Human lymphoblastoid cells (Namalva)	50
8	Mouse L cells (monolayer) subconfluent to 8-day confluent	0.6–6
9	†Green monkey kidney cells (Vero): subconfluent to 2-day confluent	0.1–1
10	Human diploid fibroblasts (MRC5): passage 37, subconfluent to 6-day confluent	<0.1–0.3
11	†Pig lymphocytes	1.5*
	Phytohaemagglutinin-stimulated	3*
12	† <i>Xenopus laevis</i> :	
	liver	<0.04
	pooled blood cells	<0.04
13	† <i>Drosophila melanogaster</i> :	
	20-h embryos	<0.04
	3rd instar larvae	<0.04
	adult flies	<0.04

Extracts (post-mitochondrial supernatant fractions) were prepared at 0–4 °C from packed cells or finely minced tissue which had been frozen at –70 °C as described previously⁹ except that the homogenisation buffer contained 0.5% NP40 where indicated (†). (Control experiments showed 0.5% NP40 to be without effect on the assay of the synthetase.) The absorbance of the extract at 260 nm (A₂₆₀) was determined after dilution with 0.4 M NaOH. The synthetase was bound to poly(I) · poly(C)-paper by incubating 100–200 µl of extract with 1 cm² of paper for 1 h at 25 °C. The paper was washed thoroughly with 90 mM KCl, 10 mM HEPES, pH 7.5, 1.5 mM magnesium acetate, 7 mM 2-mercaptoethanol, 20% glycerol (v/v). It was incubated for 30 min at 25 °C with 1 ml of the same buffer containing 50 mM KCl, and then for 18 h at 30 °C with 10–200 µl of this buffer containing 3 mM ATP and 8.5 mM magnesium acetate. The yield of 2-5A was assayed by determining the dilution required for 50% inhibition of protein synthesis in an L-cell-free system programmed with encephalomyocarditis virus RNA, in comparison with a standard preparation of 2-5A assayed in parallel^{4,11,15}. Specific activities are based on the amount of 2-5A produced per h, in AMP equivalents. Poly(I) · poly(C)-paper (10–20 µg poly(I) · poly(C) per cm²) was prepared by a modification of the method of Alwine *et al.*¹⁶. To a 20 × 10 cm sheet of diazobenzoyloxymethyl-paper were added 5.0 ml of a mixture of 0.5 ml poly(I) · poly(C) (10 mg ml^{–1}), 0.9 ml of 25 mM sodium phosphate buffer, pH 6.0 and 3.6 ml dimethyl sulphoxide. After incubation overnight at room temperature, the following mixture was added to allow the denatured RNA to rehybridise: 3 ml bovine serum albumin (2 mg ml^{–1}, pretreated with 0.1% diethylpyrocarbonate for 1 h at room temperature), 1.5 ml 10% SDS, 25 ml 2 × SSC (300 mM NaCl, 30 mM trisodium citrate); 30 ml formamide, 8.5 µl poly(C) (5 mg ml^{–1}). After 24 h at room temperature with gentle shaking, the paper was washed thoroughly 3 times with 2 × SSC, blotted dry and stored at –70 °C. Interferon treatment of mouse L cells was as described previously²; treatment of the human diploid fibroblasts (ICRF23) was for 24 h at 37 °C with 10³ units ml^{–1}.

* The A₂₆₀ of these extracts was not determined and was assumed to be 100 for the calculation of specific activity. Values of 20 to 160 were determined for the remainder of the samples.

interesting but its significance is not known. More interestingly, the level of enzyme in chick oviducts varied with the time of withdrawal from stimulation by oestrogen (see below and Table 2). A preliminary search for a similar phenomenon in mammary glands from lactating guinea pigs, which involute on removal of the young, has yielded equivocal results so far (R. K. Craig, G.R.S., R.E.B. and I.M.K., unpublished). Finally, in a total of six experiments, enzyme levels were compared in subconfluent and confluent monolayers of L, Vero and MRC5 cells. The level of enzyme in confluent cells increased very gradually for up to 8 d.

In the experiments with chick oviducts, despite some individual variations (see Table 2 legend), the same trend was seen in multiple assays of extracts from five different sets of oviducts. It seems reasonable to conclude, therefore, that the assayable enzyme remains relatively low in the first 24 h after hormone withdrawal and increases gradually thereafter, to reach final levels about 10-fold higher, approaching those in reticulocytes or interferon-treated cells (Table 1). Control experiments indicated that the differences observed with extracts made as described in Table 2 probably reflect changes in the level of 2-5A synthetase rather than changes in the levels of an antagonist or degradative enzyme. For example, a dominant inhibitor was not detected in mixing experiments. In addition, synthesis of 2-5A was linear for up to 40 h with paper-bound enzyme from oviducts early (1 h) or late (10 d) after withdrawal from hormone, and added 2-5A was equally stable in either case.

The inhibitor of protein synthesis generated by incubation of the enzyme bound to poly(I) · poly(C)-paper was not characterised in most of the experiments shown in Table 1. However, the product from chick oviducts was radioactively labelled¹¹ and characterised as 2-5A by thin-layer chromatography, analysis on DEAE-cellulose in the presence of 7M urea^{4,7}, and high pressure liquid chromatography. It also had the same specific biological activity as mouse or rabbit reticulocyte 2-5A in inhibiting cell-free protein synthesis.

These results demonstrate the wide distribution and variation in level of detectable 2-5A synthetase activity in a variety of cells and tissues. Small differences in activity are probably not significant with the biological assay used, but it seems improbable that the large differences observed in Tables 1 and 2 are trivial. The relationship between the increase in 2-5A synthetase and the decrease in the proportion of tubular gland cells which accompanies long-term withdrawal of the oviduct from oestrogen stimulation is not known, but the enhanced level of synthetase suggests a possible involvement of the 2-5A-dependent nuclease in the regression of this tissue. A role for the 2-5A system in the rapid degradation of ovalbumin mRNA that quickly follows withdrawal of oestrogen (ref. 14 and W.J.D. and R.T.S., unpublished) is also possible, despite the apparently low levels of 2-5A synthetase at this time (Table 2). Note that here we are assaying only one component of the 2-5A system and we have no means of knowing what levels would be rate limiting in different conditions in the intact cells. Moreover, we cannot yet be certain that pre-activated 2-5A synthetase already bound to dsRNA (or alternative activator) might not be sequestered and unavailable to the assay used, that is, here we may be assaying potentially active rather than total, or active, enzyme. 2-5A has a short half life in chick and L-cell-free systems, but saturates the activatable nuclease at very low concentrations, below 10 nM (ref. 9, B.R.G. Williams and I.M.K., unpublished data). Accordingly, to understand the operation of the 2-5A system fully it will also be necessary to determine the nature and level of the (dsRNA) activator of the synthetase, whether the level of 2-5A sensitive nuclease varies, what intracellular levels of 2-5A are achieved and how these levels are maintained by a balance of synthesis and degradation in different tissues in different conditions. This last point will require the development of a convenient quantitative assay for 2-5A capable of detecting physiological concentrations (≤ nanomolar) in extracts of cells. Meanwhile, despite the uncertainties outlined above in the

Table 2 Effect of withdrawal of oestrogen stimulation or 2-5A synthetase activity in chick oviducts

Hormonal status	Specific activity (pmol 2-5A per A ₂₆₀ per h)	
	Expt 1	Expt 2
Fully stimulated	—	12
1 h-withdrawn	6	—
3 h-withdrawn	16	—
6 h-withdrawn	13	—
12 h-withdrawn	17	—
18 h-withdrawn	17	—
1 d-withdrawn	—	12
2 d-withdrawn	—	15
3 d-withdrawn	—	62
5 d-withdrawn	—	63
7 d-withdrawn	—	70
10 d-withdrawn	134	130

Extracts prepared without NP40 from chick oviducts prefrozen at -70°C were assayed for 2-5A synthetase by the method given in Table 1, scaled down 10-fold. Oestrogen stimulation of 5 to 7 day-old chicks and withdrawal after 14 days were accomplished by subcutaneous implantation and removal of diethylstilbestrol-impregnated silastic pellets (W.J.D. and R.T.S., unpublished). There was some variability in the values obtained in different experiments: for example, 6 different 10 day-withdrawn oviducts yielded values of 85, 88, 120, 130, 134 and 200. The only extensive variation was obtained with five fully stimulated oviducts, which gave values of 1.5, 5.5, 12, 15 and 290. The reason for this variability is not known, although the one very high value might reflect an elevated interferon level due to an undiagnosed virus infection.

interpretation of the levels of synthetase detected, the very different levels observed, whether they reflect total or only potentially active enzyme, are of interest and indicate a wider significance for the 2-5A system than simply in the antiviral action of interferon. Taken together, the function of 2-5A in activating a nuclease which degrades mRNA, the widespread occurrence of 2-5A synthetase, the high levels of this enzyme in highly differentiated cells where mRNA degradation may have played a part in preparing the cell for specialised function¹² and the increase in the level of synthetase in some situations strongly suggest that the 2-5A system may have an important regulatory function in processes such as maintenance of cell stasis, tissue regression and differentiation.

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'Freezing' of Ca-regulated conformation of reconstituted thin filament of skeletal muscle by glutaraldehyde

REGULATION of skeletal muscle contraction by calcium ions is mediated by the troponin-tropomyosin system located in the groove of actin double strands¹. Acting with tropomyosin, troponin depresses the ability of the actin filament to interact with the myosin molecule in the presence of ATP. Some structural changes in the thin filament associated with this depression have been detected by physical techniques²⁻⁸. When the actin filament is cross-linked with glutaraldehyde, it retains the ability to activate myosin ATPase⁹, and troponin and tropomyosin can no longer exert their regulatory effect on these cross-linked or 'frozen' actin filaments¹⁰. Using glutaraldehyde as cross-linking agent we have now been able to 'freeze' the activated (presence of calcium) and depressed (absence) states of the actin filament induced by the troponin-tropomyosin system: this suggests that the structural changes detected using physical methods are essential for regulation by calcium.

Reconstituted thin filament, composed of 0.25 mg ml⁻¹ troponin, 0.22 mg ml⁻¹ tropomyosin and 1 mg ml⁻¹ actin from rabbit back muscle, was incubated with 0.1% glutaraldehyde (Sigma) in a solution containing 0.1 M KCl, 3 mM MgCl₂ and 50 mM HEPES (pH 7.2) for 7 min at 25 °C in the presence or absence of calcium ions. The cross-linking reaction was terminated by adding KSCN to a final concentration of 50 mM, and the resulting solution containing cross-linked thin filaments was dialysed against 0.1 M KCl containing 3 mM MgCl₂ and 20 mM Tris-HCl (pH 7.6).

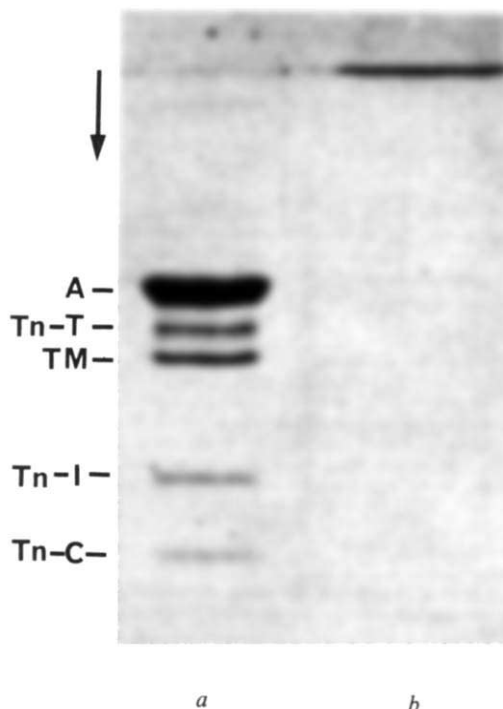


Fig. 1 SDS-polyacrylamide gel electrophoresis¹⁵ of the reconstituted thin filament before (a) and after (b) cross-linking with glutaraldehyde.

Table 1 Myosin ATPase activities activated by cross-linked thin filament

Type of thin filament	ATPase ($\mu\text{mol Pi per min per mg}$)	
	$10^{-7} \text{ M Ca}^{2+}$	$1.3 \times 10^{-5} \text{ M Ca}^{2+}$
Control	0.025	0.112
Cross-linked in the absence of Ca^{2+}	0.042	0.039
Cross-linked in the presence of Ca^{2+}	0.145	0.150

ATPase was assayed at 25°C in a solution containing 0.2 mg ml^{-1} myosin, 0.2 mg ml^{-1} thin filament, 0.1 M KCl , 3 mM MgCl_2 , $20 \text{ mM Tris-maleate}$ ($\text{pH } 6.8$) and 1 mM ATP . Ca^{2+} concentration shown corresponds to the presence of 0.1 mM EGTA alone (10^{-7} M) or the presence of 0.1 mM EGTA containing 0.095 mM CaCl_2 ($1.3 \times 10^{-5} \text{ M}$). ATPase activity was determined by the method of Youngburg and Youngburg¹⁶.

On SDS gels, most proteins of the cross-linked thin filament did not enter the acrylamide matrix, indicating that a considerable degree of intermolecular cross-linking had occurred (Fig. 1). However, under the electron microscope no differences in filamentous features could be seen between the thin filament before and after cross-linking.

As shown in Table 1, the reconstituted thin filament cross-linked in the presence of the calcium ion (that is, in the activated state) did not show the depression of actin-activated myosin ATPase even in the absence of the calcium ion, whereas the filament cross-linked in the absence of the calcium ion (in the depressed state) showed only a low level of activation of myosin ATPase even in the presence of calcium ion. In each case, the ATPase activity was not influenced by the calcium ion.

Thus cross-linking with glutaraldehyde can 'freeze' the structure of the thin filament in a specified functional state. Glutaraldehyde may therefore be useful as an aid in the structural analysis of the thin filament⁸. When dimethylsuberimide dihydrochloride was used as cross-linking agent in place of glutaraldehyde, extensive cross-linking of the thin filament was observed but the interaction of the resulting thin filament with myosin was still regulated by calcium ions.

The method described here will be of wide application, and has already provided evidence to show that gizzard muscle regulation¹¹ is actin-linked, as has been asserted by Ebashi *et al.*¹²⁻¹⁴.

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Movement of myosin heads during a heart beat

X-RAY diffraction studies of heart muscle undergoing cyclic contractions have shown that the myosin heads are transferred from the vicinity of the thick filaments to that of the thin filaments as the muscle passes from the diastolic (relaxed) to the systolic (contracted) state^{1,2}. We have now followed the time course of this transfer using a fast X-ray diffraction technique and found that the transfer occurs almost in parallel with tension development.

A papillary muscle was isolated from the right ventricle of a canine heart and cross-perfused with the arterial blood of a donor dog³. A branch of the arterial tubing was used to drip warm blood on to the surface of the muscle to keep the temperature of the muscle at about 37°C . The muscle was held isometrically at the optimal length (L_{max}) and was stimulated once a second with a square pulse of 5-ms duration (for details see ref. 4). The muscle was connected to a tension transducer (Shinko, UL) and mounted vertically in a low-angle camera of the mirror-monochromator type⁵. The X-ray source was a rotating-anode generator (Rigaku FR) operated at 50 kV with a tube current of 70 mA (the size of the focal spot was $1 \times 0.1 \text{ mm}$). The equatorial diffraction pattern from the muscle was recorded with a position-sensitive counter of the triangular-cathode type⁶ at a specimen-to-counter distance of 70 cm. The output of the counter was fed to a memory circuit⁷⁻⁹ which registered separately the signals from 16 different phases during the cardiac cycle. To obtain reasonable photon statistics for each phase the cardiac cycle was repeated 4,000-10,000 times.

The muscle produced a peak tension of $483 \pm 38 \text{ g cm}^{-2}$ at a time $250 \pm 8 \text{ ms}$ after the beginning of the stimulus (mean $\pm$ s.e.m., number of muscles = 5). The diffraction pattern showed the 1, 0 and 1, 1 equatorial reflections from the hexagonal array of the myofilaments (Fig. 1). We measured the integrated intensities of these reflections and calculated the intensity ratio ($I_{1,0}/I_{1,1}$). On contraction the 1, 0 intensity decreased and the 1, 1 intensity increased, so that the ratio $I_{1,0}/I_{1,1}$ decreased.

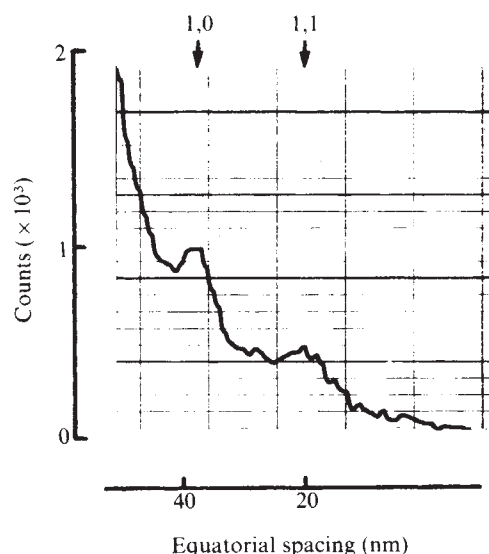


Fig. 1 Equatorial intensity distribution (right half only) of the X rays scattered by a canine papillary muscle in a 62.5-ms period during the diastolic phase. Thin papillary muscles ($0.65\text{--}1.2 \text{ mm}$ thick) were chosen in order to avoid high background scatter. The muscles were capable of contracting more than 20,000 times with little decline of peak tension⁴. Approximately 4,800 contractions were needed for recording the intensity distribution. The intensities of the 1, 0 and 1, 1 reflections from the hexagonal myofilament lattice were obtained by measuring the area under the peaks; the background level under each peak was drawn in by eye.

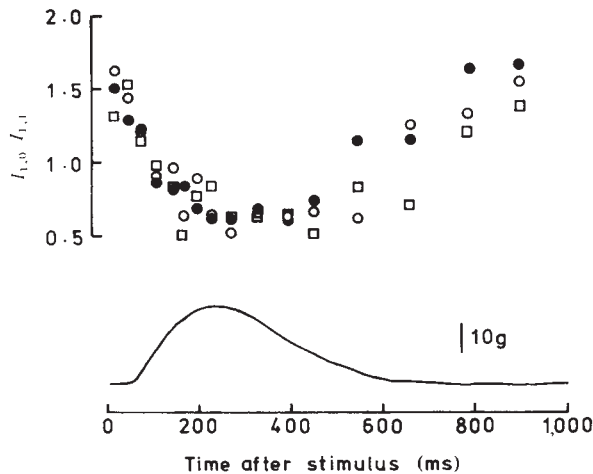


Fig. 2 Changes in the equatorial intensity ratio ($I_{1,0}/I_{1,1}$) in a papillary muscle during the cardiac cycle. The 1-s cycle was divided into 16 phases, and the ratio was measured for each phase; the first 250 ms of the cycle was divided into eight 31.25-ms phases, the following 250 ms into four 62.5-ms phases, and the last 500 ms into four 125-ms phases. Note that, as the contraction was cyclic, the last period of measurement (875–1,000 ms after the beginning of the stimulus) was followed immediately by the first period (0–31.25 ms after the beginning of the stimulus). The same set of diffraction patterns was measured by three different people who made their own estimation of the background level (see legend to Fig. 1); symbols ($\circ$, $\bullet$, $\square$) represent the measurements by different people. The tension record was obtained by averaging 10,000 contractions with a multichannel analyser⁹. The cross-sectional area of the muscle at its middle was 0.045 cm^2 .

Figure 2 shows that the time course of the change in intensity ratio was an approximate mirror image of the tension curve; the ratio reached the minimum value of 0.74 ± 0.06 ($n = 5$) at approximately the same time as the peak tension.

The observed decrease in the intensity ratio was caused partly by radial transfer of myosin heads from the thick to the thin filaments and also by slight shortening of sarcomeres; although the muscle was held isometrically, the sarcomeres in the middle of the muscle became about 10% shorter at the peak of contraction². Figure 3 shows the result of an experiment in which we assessed the contribution of the sarcomere shortening. First we obtained an intensity-ratio time-course with a muscle length of $L_{\max}$ (solid line). Then we repeated the measurements (dotted line) on the same muscle with a muscle length of $0.9L_{\max}$ so that the sarcomeres were already 10% shorter in the diastolic phase (and therefore about 20% shorter at the peak of contraction). If the sarcomere length had stayed constant (at the length which was attained at the peak of contraction for the first set of measurements) throughout the cardiac cycle, the intensity ratio would have changed in the following manner: (1) the ratio would have corresponded to a point on the dotted line at the beginning of the cycle, and moved toward the solid line as the tension developed; (2) the ratio would have reached the solid line at the peak of tension; the minimum ratio at a constant sarcomere length would have been found near the minimum of the solid line; and (3) the ratio would have moved toward the dotted line during the falling phase of tension, reaching the dotted line when the tension reached the steady diastolic level. Such an imaginary curve would represent more correctly the radial transfer of myosin heads; a decrease in the ratio at a constant sarcomere length represents an increase in the number of heads in the vicinity of the thin filaments^{10,11}. Our results suggest that the number reaches a maximum approximately at the time of peak tension, at ~ 250 ms after the stimulus at 37°C . This is much slower than in skeletal muscle in which the number reaches a maximum within 70 ms after the stimulus at 0°C and even earlier at higher temperature^{9,12} (the peak tension in skeletal muscle occurs 150–160 ms after stimulus at 0°C). The slow

transfer of myosin heads in heart muscle may be the basis of the slow development of the active state in heart muscle¹³.

The maximum intensity ratio obtained during the diastolic phase was 1.72 ± 0.09 ($n = 5$) at $L_{\max}$, and smaller at $0.9L_{\max}$ (Fig. 3). It is known that a quiescent heart muscle at $0.9L_{\max}$ gives an intensity ratio of 3.07 (ref. 1) which is considerably greater than the maximum ratios obtained in the present study. This indicates that, during the cardiac cycle, the myosin heads as a whole never go into the 'quiescent' state; there is always a considerable number of heads remaining in the vicinity of the thin filaments during the diastolic phase. However, these myosin heads are unlikely to be producing significant force during the diastolic phase as a previous study² has shown that a change in the number of 'remaining' myosin heads is not accompanied by a change in the diastolic tension.

It is of interest to know how the behaviour of myosin heads during the cardiac cycle is affected by the physiological means used to change the size and the time course of tension development. We studied the effect on the intensity ratio of repetitive stimulation with paired pulses; this type of stimulation increases the peak tension and shortens the time from stimulus to the peak tension¹⁴. A muscle was first stimulated once a second with single pulses. Then the same muscle was stimulated every second with a pair of pulses to cause stronger contractions. The interval between the pair of pulses was set at 270 ms to produce maximal potentiation. The time course of the intensity ratio for the two patterns of stimulation are compared in Fig. 4. When stimuli were changed from single to paired pulses, the muscle had to be stretched slightly in order to re-adjust its length of $L_{\max}$; the internal shortening of sarcomeres was enhanced by the increased tension. This re-adjustment allowed us to compare the minimum intensity ratios, which were obtained at the time of peak tension both with single and paired pulses, at approximately the same sarcomere length. The minimum ratio was smaller with paired pulses than with single pulses, suggesting that paired-pulse stimulation increases the number of myosin heads found in the vicinity of the thin filaments at the peak of contraction. This is likely to be the basis of the tension increase caused by paired pulses. The intensity ratios in the diastolic

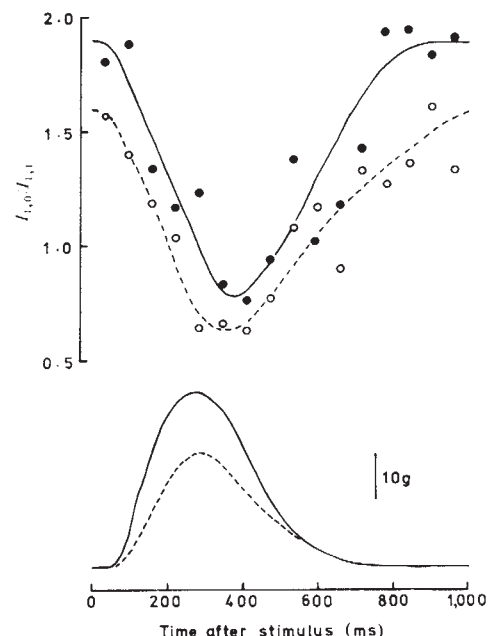


Fig. 3 The intensity ratios obtained from the same muscle at different muscle lengths, $L_{\max}$ (filled circles and solid line) and $0.9L_{\max}$ (open circles and dotted line). The 1-s cardiac cycle was divided into 16 phases of 62.5-ms duration. The tension records are averages of all the contractions needed for recording diffraction patterns at $L_{\max}$ and $0.9L_{\max}$. The cross-sectional area of the muscle at $L_{\max}$ was 0.084 cm^2 .

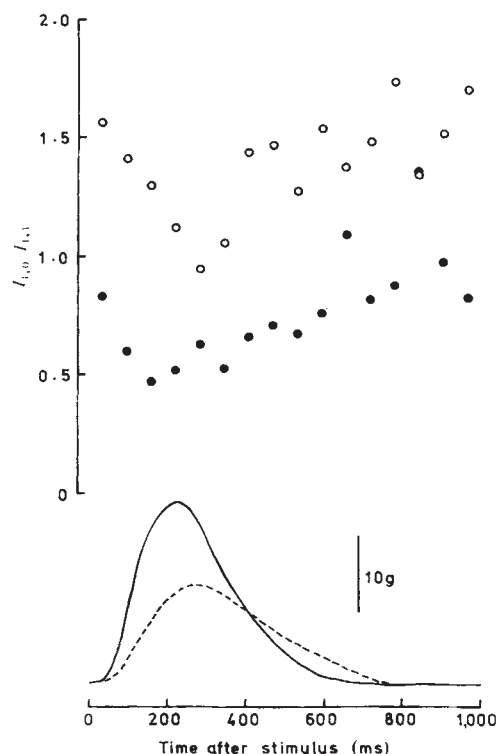


Fig. 4 The intensity ratios obtained from the same muscle contracting at the same frequency (1 s^{-1}) but with different types of stimuli; single pulses ($\circ$) and paired pulses ($\bullet$). The interval between the pair of pulses (270 ms) was in the range where maximal potentiation was obtained. The tension records are averages of all the contractions needed for recording diffraction patterns with single pulses (dotted line) and paired pulses (solid line). The cross-sectional area of the muscle was 0.023 cm^2 .

phase were also smaller with paired pulses than with single pulses. This decrease in the ratio was not attributable to the re-adjustment of muscle length as stretching a muscle would be expected to increase the ratio (Fig. 3). Therefore, our observation suggests that the number of myosin heads remaining in the vicinity of the thin filaments during the diastolic phase is also increased by paired-pulse stimulation.

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Tn554—a site-specific repressor-controlled transposon in *Staphylococcus aureus*

PREVIOUS studies have defined a specific element in *Staphylococcus aureus* containing determinants of inducible erythromycin resistance and spectinomycin resistance. This element has many plasmid-like properties^{1,2} but is not associated with detectable extra-chromosomal DNA³. The resistances are similar in mechanism to those ordinarily associated with plasmids^{3,4} (J. Davies, personal communication); the element can be transferred by transduction² or transformation to a *rec*⁻ recipient with the same efficiency as to a *rec*⁺, and the two determinants show essentially 100% linkage in genetic transfer. Consequently, this linkage group has previously been referred to as a 'pseudoplasmid' (ref. 2). On the basis of the results presented here, we suggest that this pseudoplasmid is actually the prototype of a new class of transposon, which functions by means of a highly efficient, repressor-controlled, site-specific integration-excision mechanism. This transposon, designated Tn554 (EmSp), can be most easily envisaged as a prophage-like element that lacks replicative autonomy and other vegetative phage functions.

Table 1 lists the *S. aureus* strains used. Transduction and transformation were carried out by standard methods^{5,6} whole-cell and plasmid DNA were isolated and analysed by standard methods^{6,7}.

Initial transformation mapping results indicated that Tn554 is weakly (co-transformation frequency of $\leq 1\%$) but definitely linked to the chromosomal *tmn* (tetracycline-minocycline resistance) locus previously mapped by Pattee and Neveln⁶. Concurrently, mapping of a series of chromosomal insertions of a different transposon, Tn551 (ref. 8), suggested that one of these, $\Omega 34$, was located at a site very near to that of Tn554 (ref. 9). Accordingly, we carried out a series of transductions to test for linkage between the two, and found that Tn554 and $\Omega 34$ are indeed very closely linked (Table 2). Somewhat inconveniently, Tn551, first found on plasmid pI258 (ref. 8), also encodes erythromycin resistance (in this case, constitutive). Its properties are similar to those of the various *Escherichia coli* transposons¹⁰: it translocates with a frequency of about 10^{-4} per donor genome and inserts at many different chromosomal and

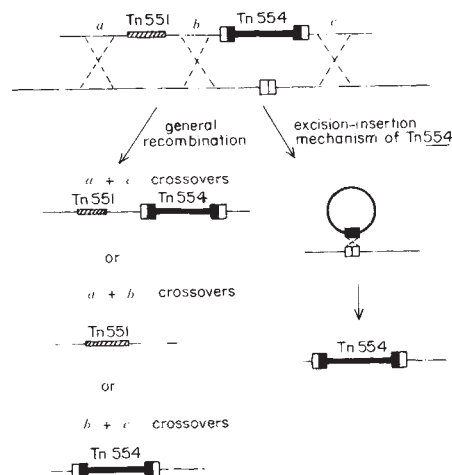


Fig. 1. Possible mechanisms for Tn554 integration. Thin lines represent chromosomal sequences; hatched bar represents the $\Omega 34$ insertion of Tn551; solid bar represents Tn554; open blocks represent the putative attachment site of Tn554; dashed lines represent possible homologous crossovers, resulting in the insertion of the entire region ($a + c$), of Tn551 only ($a + b$), or of Tn554 only ($b + c$). In addition, Tn554 can evidently excise and insert independently; this is presumably its primary mode of transfer to a *rec* recipient.

Table 1 Staphylococcal strains and plasmids

Plasmids		Genotype*	Derivation
Plasmid no.			
pRN1107		pI524 <i>repA2</i> Δ 137 [<i>bla</i> <i>asa</i> <i>asi</i>]	pI524 <i>blaII</i> <i>repA2</i>
pRN4166		Ω 171 [<i>pRN1107::Tn554 ermII</i> <i>spc-1</i>]	pRN1107
Strains	Host strain	Chromosomal markers	Derivation
stock no.			
RN27	8325-3 (80 α)		RN25, 80 α lysogen
RN496	8325-4	Ω 5 [<i>chr::Tn551</i>]	
RN1030	8325-4 (ϕ 11)	<i>recA1</i>	
RN2573	8325 (80 α)	Ω 34 [<i>chr::Tn551</i>]	
RN2784	8325-4	Ω 34 [<i>chr::Tn551</i>]; [<i>chr::Tn554 ermA4</i>]	Ω 34 $\rightarrow$ RN2867
RN2863	8325-4	<i>chr::Tn554 ermII</i> <i>spc-1</i>	
RN2870	8325-4 (ϕ 11)	<i>chr::Tn554 ermA5</i>	<i>Tn554 ermA5</i> $\rightarrow$ RN451
RN2896	8325-3 (80 α)	<i>chr::Tn554 ermA5</i>	<i>Tn554 ermA5</i> $\rightarrow$ RN27
RN3078 to RN3082	8325-4 (ϕ 11)	<i>recA1</i> [<i>chr::Tn554 ermA5</i>]	<i>Tn554 ermA5</i> $\rightarrow$ RN1030

* Key: Ω , insertion; Δ , deletion; $\rightarrow$, transfer.

plasmid sites. Although its transposition is *rec*-independent, it cannot be transduced at a detectable frequency from a chromosomal site to a *rec*⁻ recipient². This is presumably merely a question of multiplied probabilities; a transposition frequency of 10⁻⁴ coupled with a transduction frequency of 10⁻⁶–10⁻⁷ results in a combined probability of <10⁻¹⁰, which is well below the limits of detectability in this system.

Transduction with RN2784, carrying Ω 34 and an Em^s mutant of *Tn554*, as donor, revealed 50% linkage between *Tn554* and Ω 34 when selection was for the *Tn551* Em^r determinant, but only 10% when selection was for the *Tn554* Sp^r determinant. This asymmetry could either be due to transductional polarity (involving the specificity of transducing fragment ends)¹¹, or to specific excision–insertion of *Tn554*, which inevitably disrupts the linkage with the Ω 34 locus. Two lines of evidence support the latter interpretation. In a similar experiment carried out by transformation, the same asymmetry was obtained; and in a transductional cross using the same donor but a *rec*⁻ recipient, no co-transduction of *Tn554* and Ω 34 was observed; all of 315 Sp^r transductants were Em^s and no Em^r transductants were obtained. Further, in these experiments, the entire *Tn554 ermA4* element was acquired; all of five Em^sSp^r transductants tested reverted to Em^r (inducible) at the same frequency as the original *Tn554 ermA4*.

On the basis of these results, it seemed possible that specific excision–insertion of *Tn554* was responsible for its transfer to the *rec*⁻ recipient. Two other possibilities were considered: (1) that it was maintained as a plasmid in the *rec*⁻ strain, and (2) that it carried the *rec*⁺ allele, enabling it to recombine into the chromosome. These were ruled out by a failure to demonstrate plasmid DNA in two different *rec*⁻ transductants and by a demonstration that these were unable to accept the chromosomal *tmn* marker and were, therefore, still *rec*⁻. Because transfer of *Tn554* to a *rec*⁺ recipient could involve either homologous recombination or specific insertion, whereas transfer to a *rec*⁻ could involve only the latter, the site specificity of the putative insertion process was tested with several independent *rec*⁻ transductants. *Tn554* was mapped in the *rec*⁻ by scoring for transductional displacement of *Tn551* in *rec*⁺ recipients containing Ω 34 in comparison with Ω 5, a *Tn551* insertion unlinked to *Tn554* by transduction. Five different *rec*⁻ *Tn554* strains each gave about 8% displacement of Ω 34 but no detectable displacement of Ω 5, consistent with insertion of *Tn554* in the *rec*⁻ chromosome at the same location as in the *rec*⁺ (Table 2).

The high frequency of excision and insertion required to account for the efficient transfer of *Tn554* to the *rec*⁻ strain, and the very great stability of the element once established, suggested that excision, at least, is controlled by a transposon-specific repressor. If so, the high frequency of transfer to the *rec*⁻ would be analogous to zygotic induction, as the recipient would lack repressor; that is, the element would excise from the transducing

fragment on which it was injected by the phage, and then insert by site-specific recombination into the chromosome. A probable mechanism for this sequence of events is that proposed originally by Campbell¹² to account for the excision and insertion of coliphage λ (Fig. 1). The excision step could, of course, occur in the donor during phage maturation, in which case one need not postulate a repressor to account for the results described so far. In any case, as a preliminary test for the presence of a repressor, we compared the transduction frequency of *Tn554 ermII* *spc-1* to recipient strains with and without *Tn554 ermA5* and found that the presence of the *Tn554* derivative in the recipient markedly inhibited super-transduction. This inhibition was greater in the *rec*⁻ recipient (about 100-fold) than in the *rec*⁺ (about 10-fold), which is expected regardless of the mechanism of inhibition, as recombinational displacement occurs in the *rec*⁺ but not in the *rec*⁻. Although this result is consistent with the existence of a repressor of excision or of

Table 2 Site specificity of *Tn554* insertions in the *S. aureus* chromosome

Donor	Recipient	Selection	Transductant classes (%) [*]		
			Em ^r Sp ^s	Em ^s Sp ^r	Em ^r Sp ^r
RN2573	RN2896	Em ^r	64.5 (200)	—	35.5 (200)
RN3078	RN2573	Sp ^r	—	7.8 (90)	92.2 (90)
RN3079	RN2573	Sp ^r	—	10.8 (120)	89.2 (120)
RN3080	RN2573	Sp ^r	—	8.7 (160)	91.3 (160)
RN3081	RN2573	Sp ^r	—	6.0 (100)	94.0 (100)
RN3082	RN2573	Sp ^r	—	7.0 (100)	93.0 (100)
RN496	RN2870	Em ^r	0 (100)	—	100.0 (100)
RN3078	RN496	Sp ^r	—	0 (100)	100.0 (100)
RN3079	RN496	Sp ^r	—	0 (100)	100.0 (100)
RN3080	RN496	Sp ^r	—	0 (100)	100.0 (100)
RN3081	RN496	Sp ^r	—	0 (100)	100.0 (100)
RN3082	RN496	Sp ^r	—	0 (100)	100.0 (100)

* Transductants per plaque-forming unit of phage (PFU) were approximately 10⁻⁶ for *Tn551* donors and 10⁻⁵ to 10⁻⁶ for *Tn554* donors.

Figures in parentheses represent numbers of transductants scored for this value. Key: Em^r = erythromycin resistant; Sp^r = spectinomycin resistant; Em^s = erythromycin sensitive; Sp^s = spectinomycin sensitive.

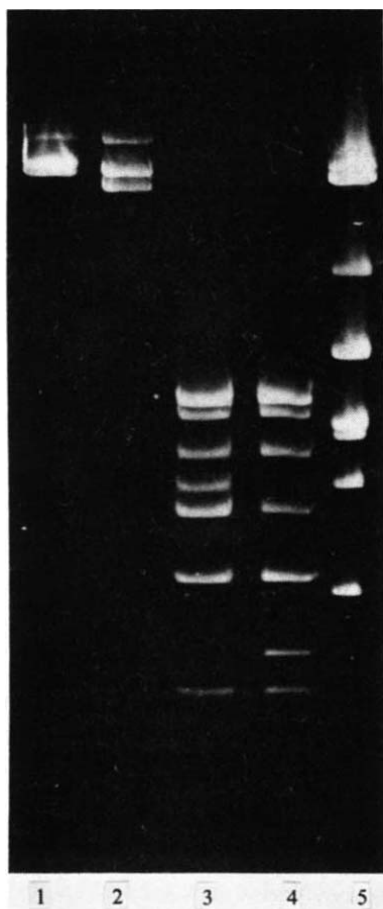


Fig. 2 Agarose (0.7%) gel electrophoresis of plasmids digested with *Bam*HI and *Bgl*II. In each pair, the digest of the plasmid carrying the *Tn554* insertion is on the left. Slot 1 = pRN4166 cut with *Bam*HI; slot 2 = pRN1107 cut with *Bam*HI; slot 3 = pRN4166 cut with *Bgl*II; slot 4 = pRN1107 cut with *Bgl*II. Slot 5 contains phage λ *Eco*RI fragments. Note the loss of a 2.6-kilobase pair *Bgl*II fragment (slot 4) and the appearance of two new fragments, one of which co-migrates with a fragment already present, and thus appears as a doublet (slot 3).

excision-insertion, an alternative possibility is direct interference by the resident transposon with the integration of the superinfecting one, through insertional disruption of the putative attachment site (Fig. 1).

To distinguish between these two possibilities, it was necessary to obtain a *Tn554* insertion in a secondary site, most conveniently a plasmid. This was initially attempted with a strain carrying a penicillinase plasmid (pI524) plus *Tn554* as a donor in a transduction with double selection for the plasmid-linked cadmium resistance (*Cd*^r) and *Tn554*-linked *Em*^r markers. Although a few doubly resistant transductants were obtained, these were all found to contain the original plasmid and a chromosomal *Tn554*. In retrospect, this is the expected result if, indeed, there is zygotic induction of *Tn554* transposition. As this result suggested that *Tn554* could insert into the plasmid, we adopted the approach of transducing *Tn554 ermI1 spc-1* into a *rec*⁻ recipient containing *Tn554 ermA5* in the chromosome (as the source of the putative repressor), and a plasmid, pRN1107, as a secondary target for the transposon. The plasmid used was a pI524 derivative with a thermosensitive replication defect (*Tsr*)¹³, permitting the scoring of plasmid linkage by testing for co-elimination during growth at 43°C, the restrictive temperature. As before, there was a 100-fold reduction of *Tn554 ermI1 spc-1* transduction frequency in these conditions, but in one of 10 *Em*^r transductants obtained, the incoming *Tn554 ermI1 spc-1* was co-eliminated with cadmium resistance during growth at 43°C, and was therefore presumed to have inserted into the plasmid. The apparent plasmid linkage was confirmed by

agarose gel electrophoresis after digestion with restriction endonucleases. These results revealed a 6.2-kilobase insertion in the plasmid carrying *Tn554* (Fig. 2).

Having obtained a plasmid insertion of *Tn554 ermI1 spc-1*, we were able to test more directly for the action of a repressor. This was accomplished by further transduction of this plasmid, pRN4166, into two new recipient strains, one carrying a chromosomal *Tn554 ermA5* insertion (RN2896), the other not (RN27). In this experiment, *Cd*^r (plasmid-positive) transductants were tested first for the presence of the *Tn554 ermI1 spc-1 Em*^r marker and then, by temperature-induced curing, for the linkage of *Tn554 ermI1 spc-1* to the plasmid.

The results of this test indicated that the plasmid and the inserted transposon were co-transduced with a frequency of about 50% (the reason for this is now under investigation); among the co-transductants obtained, the linkage between the plasmid and the transposon was disrupted in all of 10 *Cd*^r*Em*^r clones derived from the recipient lacking a chromosomal *Tn554* and was preserved in all of 10 derived from the recipient containing one. Here again, the linkage was evaluated by temperature-induced curing of the plasmid.

This result is consistent with a repressor of *Tn554* excision encoded by the transposon itself. If site-specific interference with insertion were responsible for the inhibition of transduction described above, then the chromosomal *Tn554* element should not have had any effect on excision of the incoming transposon from the plasmid, only on its subsequent insertion into the chromosome. It is conceivable, however, that excision and insertion are integrated processes, so that if insertion is blocked, excision is automatically blocked also. We consider this unlikely because excision regenerates an intact plasmid; this suggests that *Tn554* is excised in the double-stranded configuration.

The simplest mechanism in which double-stranded excision is coupled with restoration of continuity is that proposed by Campbell for the excision-insertion of coliphage λ (ref. 12). In this mechanism, involving the generation of a circular intermediate by a site-specific intramolecular crossover, excision occurs independently of the fate of the excised molecule. If this is, in fact, the correct interpretation for *Tn554* transposition, then it is the first example of transposition occurring by the Campbell mechanism. We note that other transposons, particularly *Tn9*(Cm), also show *rec*-independent excision with preservation of the carrier genome, but there is no evidence for a *Tn9* repressor, and the excised transposon is nearly always lost rather than reinserted at another site¹⁴.

Figure 1 is a summary of the postulated mechanisms of transfer and insertion of *Tn554*. Experiments are now in progress with the aim of confirming the existence of a repressor and analysing the excision-insertion process it controls.

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matters arising

Early Precambrian oxygen

FEW of the arguments presented by Towe¹ seem strong enough to build up a case against the photosynthetic origin of Precambrian oxygen as defended, for example, by Margulis *et al.*². Note especially that he does not discuss the relative importance of hydrogen escape compared with recombination after water photolysis although it seems to be a critical issue. To the uninformed, however, one argument might seem decisive: the requirement of oxygen for chlorophyll-*a* biosynthesis. If this has been proved absolute it would be difficult not to admit the existence of some free oxygen before the formation of that molecule necessary for oxygen releasing photosynthesis.

Towe has cited Bogorad³ whose Fig. 1, which summarises chlorophyll-*a* biosynthesis, shows an oxygen-requiring step in the oxidative decarboxylation of coprophenol. This is, however, a common step to every porphyrin (haem as well as chlorophylls and bacteriochlorophylls) which in 1966 had essentially been studied with animal mitochondria. In this text (p. 492) it is mentioned that oxygen cannot be universally required for that reaction as it suppresses bacteriochlorophyll production in the purple non-sulphur bacterium *Rhodospirillum rubrum*. In a more recent treatment of this subject⁴, Bogorad indicated in his Fig. 2 that an alternative to oxygen exists for that step and gives full reference to the work of Tait⁵ who showed that photosynthetic bacteria have both aerobic and anaerobic coprophenolase activity. Since some blue-green algae are clearly able not only to survive but to sustain abundant growth in anaerobic conditions with photosynthesis based on reduced sulphur like some photosynthetic bacteria⁶⁻⁸, those species must be equally able to decarboxylate coprophenol without oxygen. Towe seems to consider that the anaerobic capacity of blue-green algae favours his hypothesis. I would rather consider that it also agrees with the reverse hypothesis, stating like Brock⁹ that "If the bulk of the atmospheric O₂ is indeed of biogenic origin, the organisms first involved in O₂ formation would of course have developed in an anaerobic environment and would have had to be able to live anaerobically".

The argument that in a reducing atmosphere there would be no selection pressure to tap the immense hydrogen resources of water is unconvincing. What would be the difference between

competition for dwindling reductants whether those are consumed by an oxidising atmosphere or prokaryotic photosynthesisers? Anyway even admitting immense resources of H₂S or other reductants which could be used for bacterial photosynthesis, in the competition between organisms, depletion can be a phenomenon localised to some biotic environments.

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TOWE REPLIES—Demoulin is disturbed primarily by my statement¹ that unless the molecular oxygen requirement of chlorophyll-*a* had been a later modification, the evolution of this pigment in an anaerobic early Precambrian environment would have been unfavourable. The ability of some extant blue-green algae to sustain abundant growth in reducing conditions certainly implies that they can make the necessary decarboxylation step without free oxygen. Whether or not such anaerobic decarboxylation of chlorophyll-*a* took place in early Precambrian ancestral forms having photosystem I only, is unknown. But if the sequence data of Schwartz and Dayhoff² are correctly interpreted the relevance of this point may be moot. After all, if, as they say, the evolutionary development of aerobic respiration preceded that of photosystem II (and hence oxygenic photosynthesis) the oxygen requirements of chlorophyll-*a* biosynthesis would be of limited significance.

On the other hand, the ability of some modern blue-green algae to thrive in reducing environments while endowed with photosystem II but using photosystem I only³⁻⁵ is certainly not, as Demoulin has asserted also in agreement with the reverse hypothesis; namely, that photosystem II evolved in a reducing environment. The algae in question³ may

survive anaerobic conditions using their photosystem II but they can do so only in the absence of suitable reductants, and tolerance for anaerobic conditions is not the same as dependence on such conditions⁶. There are, it is true, other blue-green algae living today that can grow in a reducing environment⁷ but these require photosystem II at all times. Here the exogenous reductants (H₂S) keep the *pO*₂ low rather than to act as primary electron donors for photosystem I. Thus it is important to distinguish between not only the different algal metabolisms but also between the effects of an anaerobic environment and those of an anaerobic-reducing environment. The point is that even today in the presence of reducing conditions, water is not the photosynthetic electron donor of choice for those blue-greens able to choose between photosystem I and II³⁻⁵. There is no such choice for the other modern blue-greens⁷ and, of course, no choice was available to any of the early Precambrian ancestors equipped only with photosystem I, energetically unable to take advantage of the hydrogen resource of water regardless of its immensity and ready availability. Thus I emphasised¹ that "only by removal of reduced substances as potential electron donors for photosystem I would there have been significant pressure (to) develop photosystem II".

Certainly, the effectiveness of atmospheric dissociation is important to the early Precambrian environments, and I stated¹ as much, but there are simply too many debatable assumptions involving the solar constant, water vapour mixing ratios, and, especially, hydrogen sources and sinks, implicit in the calculations to make any answer definitive. Photodissociation cannot be ruled out as a likely source of free oxygen in the early Precambrian.

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Effect of prostaglandin E₂ on prostacyclin production by endothelial cells

IT has been suggested by Tomasi *et al.*¹ that prostaglandin (PG) E₂ inhibits the synthesis of PGI₂ (prostacyclin) by endothelial cells. This conclusion was based mainly on their observation that the inhibition of platelet aggregation produced by a suspension of isolated hepatic cells (described as "endothelial-rich") was reduced when the hepatic cell suspension was preincubated for 10 min with 1.5–3 μ M PGE₂. An alternative explanation for this effect, apparently not considered by Tomasi *et al.* is that PGE₂ interferes with the action of PGI₂ rather than its synthesis. We therefore investigated the effects of PGE₂, first on PGI₂ synthesis by aortic endothelial cells, and second, on the inhibition of platelet aggregation caused by exogenous PGI₂.

Production of PGI₂ was measured by radioimmunoassay (RIA) of 6-oxo-PGF_{1 α} (the stable metabolite of PGI₂) in the medium above monolayers of cultured endothelium. In this RIA, 2 μ M PGE₂ showed appreciable cross reactivity, and all samples were therefore treated with 0.3 M KOH in methanol to convert PGE₂ to PGB₂, which showed very little cross reactivity. This treatment did not affect the immunoreactivity of 6-oxo-PGF_{1 α} . The production of 6-oxo-PGF_{1 α} was not reduced by 2 μ M PGE₂ (Table 1); in fact, samples incubated with PGE₂ showed some increase in the amount of immunoreactive 6-oxo-PGF_{1 α} present. Similar increases were, however, measured when 2 μ M PGE₂ was added at the end of the incubation or when 2 μ M PGE₂ was assayed in the same volume of medium alone. Figure 1 shows that as little as 0.1 μ M PGE₂ abolished the inhibition of arachidonic acid-induced platelet aggregation produced by 20 nM PGI₂.

On the basis of these results we conclude that PGE₂, even at the relatively

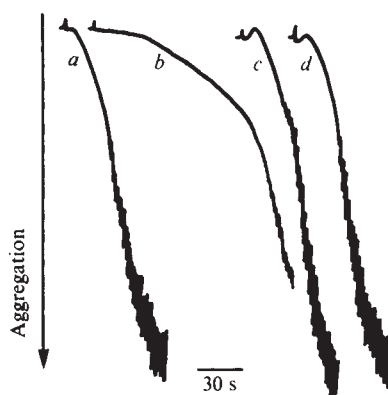


Fig. 1 Effect of PGE₂ on the inhibition of platelet aggregation caused by PGI₂. Platelet aggregation was measured photometrically in 0.1-ml samples of human citrated platelet-rich plasma⁸. Aggregation was induced by 1 mM arachidonic acid alone (a) or 1 min after the addition of 20 nM PGI₂ (b). 0.1 μ M PGE₂ was added 1 min before arachidonic acid either alone (c) or with 20 nM PGI₂ (d).

high concentration of 2 μ M, does not block PGI₂ synthesis by aortic endothelial cells, but that a lower concentration of PGE₂ can overcome the inhibitory effect of PGI₂ on platelets. The action of PGE₂ may result from competition at the platelet receptor binding PGI₂, although this seems unlikely, as we have evidence that PGE₂ and PGE₁ act at different receptors on the platelet², and PGI₂ apparently acts at the same receptor as PGE₁ (ref. 3). Alternatively, this action could be a consequence of the ability of PGE₂ to facilitate platelet aggregation induced by several stimuli, including products of arachidonate metabolism⁴. Although the hypothesis advanced by Tomasi *et al.*¹ may be valid for the hepatic cell fraction they used (although this still remains to be unequivocally established), it does not apply generally to vascular endothelium.

RIA was from Drs F. Dray and H. Sors, and PGI₂ was from Dr P. L. Walton.

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TOMASI *ET AL.* REPLY—We had considered the possibility that PGE₂ interferes with the PGI₂ receptor, and experiments showing that PGE₂ at the concentrations used (1.5–3.0 μ M) was unable to counteract PGI₂ (2–4 ng ml⁻¹) inhibition in platelet-rich plasma aggregated with sodium arachidonate, were carried out. We have now repeated these experiments with two different sources of PGI₂ with similar findings. However, we were surprised to observe that in the conditions described by Gordon *et al.* (0.1 μ M PGE₂) the results were similar to those reported in their Fig. 1. We agree that this finding cannot be explained by an antagonism of PGE₂ on the PGI₂ receptor. The most likely explanation is that at low doses (0.1 μ M) PGE₂ decreases cyclic AMP levels in platelets¹, counteracting the increase due to PGI₂ (ref. 2) and facilitating aggregation, whereas at higher doses, when PGE₂ has no effect or increases cyclic AMP¹, its action on PGI₂ inhibition is minimal or absent. Thus, in our conditions the effect of PGE₂ must be explained on the basis of a reduction of PGI₂ synthesis. This is supported by experiments showing that our preparations preincubated with PGE₂ and washed twice with buffer were much less effective than controls in inhibiting arachidonate-induced aggregation.

The failure of Gordon *et al.* to observe an effect of PGE₂ on 6-keto-PGF_{1 α} formation may be accounted for: (1) by assuming that their endothelial cells from pig aorta do not contain a PGE₂-sensitive adenylate cyclase (which would be interesting to verify), or (2) by the possibility that PGI₂ is degraded mainly to 6,15-diketo-PGF_{1 α} (ref. 3), which may not be detectable using a 6-keto-PGF_{1 α} antiserum⁴.

We would be surprised if our hypothesis applies generally to vascular endothelium. We consider that the mechanism we have described may operate mainly inside an organ where PGE₂ produced by the major cell population would mediate a control on the endothelial cells lining the organ vasculature⁵.

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Table 1 The effect of PGE₂ on 6-oxo-PGF_{1 α} production

Treatment	6-oxo-PGF _{1α} (pg)
a Endothelial cells alone	66 ± 2
b Endothelial cells + 2 μ M PGE ₂	104 ± 13
c Endothelial cells alone; 2 μ M PGE ₂ added after incubation	95 ± 6
d 2 μ M PGE ₂ alone	22 ± 2

Figures are mean $\pm$ s.e.m. (six observations in each case) and represent pg per 10⁵ cells (or, in d, pg per sample). Porcine aortic endothelial cells⁵ were incubated in serum-free tissue culture medium for 10 min at 37°C either with (b) or without (a) PGE₂, and 6-oxo-PGF_{1 α} was measured by RIA⁶. Controls (c, d) were incubated to estimate the interference of PGE₂ in the RIA.

reviews

Angels and devils of science

R. H. Pritchard

Recombinant DNA: The Untold Story. By J. Lear. Pp. 280. (Crown: New York, 1978.) \$8.95.

THIS book contains a brief account of the discoveries and discoverers that made DNA splicing exploitable as a method of amplifying genes and creating new hybrids. It also contains a blow by blow account of the rise and fall of the conviction, and the public debate it led to, that the synthesis of hybrids from organisms whose DNAs do not naturally encounter one another is hazardous because it could lead inadvertently to the creation of new pathogens not controllable by established methods.

This hypothesis in its general form was always a highly speculative one unsupported by any evidence. Its validity was determined not by reasoned argument but by shows of hands at mass meetings and *ex cathedra* pronouncements from groups of eminent scientists. The scientific community was led into a headlong rush to impose upon itself the most stringent safety precautions against totally hypothetical hazards. It caused intelligent scientists to engage in acts of public self-immolation and to public announcements of the destruction of valuable but completely harmless strains of microorganisms. Later there was cheating to get around the regulations and sneaking to get round the cheaters. It has cost millions of dollars and millions in other currencies so far to finance containment laboratories and containment bureaucracies. It has been a damaging episode in the relationship between the scientific community and the public, and there must be important lessons to be learned from it. Readers of Lear's book are in danger of learning the wrong lesson. He is not a dispassionate reporter. He has a particular point of view which leads him to a particular interpretation of the events he records. On the other hand, he has researched his material to a considerable depth. His account of events rings true. He writes entertainingly and often draws amusing and recognisable pen portraits of the central characters. His account of the science itself is admirably lucid with few howlers. It is a book well worth reading.

John Lear doesn't question the validity of the Frankenstein-monster hypothesis. Thus the story he tells is a story of the triumph of good over evil, with the devil threatening a comeback towards the end and the final outcome even now undecided. Good is accountability of scientists to society. The devil, on the other hand, believes in unbridled licence for anyone to do whatever experiment he chooses, accountable to no-one but himself. It is a fairy story, of course, but it seems to be one that Lear believes in.

According to Lear a telephone call from one Robert Pollack to a Professor Berg in 1971 sparked off the great debate. Pollack was worried that Berg proposed to propagate the genome of the monkey virus SV40 in *Escherichia coli* by splicing it into a bacterial virus called λ . Berg subsequently abandoned the experiment. Both men later organised a conference on Biohazards in Biological Research which seems to have been devoted primarily to the hazards of handling tumour virus. The conference did not get widespread publicity inside or outside the scientific community. Lear argues that it was because the press was not alerted. Later in discussing events which really did start the ball rolling he argues that the Pollack-Berg message needed time to sink in. There is another possibility. Pollack's concern was based on a reasoned (although not necessarily valid) extrapolation from well-founded observations. SV40 produces tumours in some animals and causes transformation of human cell lines to forms with cancer-like properties. It also forms natural hybrids with adenoviruses which cause respiratory infections. Anyone familiar with these facts, with the frequency with which animal viruses form hybrids naturally, and with the uncertainty surrounding their pathogenicity, would not lightly synthesise large quantities of SV40 DNA in *E. coli* and it would not be unnatural for a virologist to express his worries to a biochemist whose background might not have alerted him to the potential hazards. But these commendable initiatives could not possibly engender an impassioned debate between the angels and devils of the scientific world. Who would act the devil? Scientists are no more mis-

chievous, no less intelligent and no more suicidal than the population at large.

A conference two years later provided a more favourable breeding ground for devils. A new and trivially simple method of gene splicing was announced. Anybody could do it. The particular and rational concern (about the lack of care in handling the DNA of suspected pathogens like animal viruses) began to merge with a general and irrational one (that any new combination of genetic material will produce organisms with unknown, unpredictable, and hence potentially hazardous properties).

Devils would soon point out that to be a hazard to the population at large the novel hybrid would not only need to be pathogenic but have a positive adaptive value. To them the probability that, for example, a random insertion of fruitfly genes into *E. coli* could produce such an organism seemed, to put it simply, rather less than the probability that a random splicing of bits of the computer tape of an automatic knitting machine programmed to produce pullovers into the tape of a machine programmed to produce sox would not only lead to the generation of a wearable garment (the pullox) but one which would capture a significant share of the market. Naive angels (who seemed to believe that a cholera epidemic could be caused by *E. coli* containing a cholera toxin gene, that diarrhoea could be caused by *E. coli* carrying a cellulase gene, and tooth decay by *E. coli* with a tooth decay gene kit from *Streptococcus mutans*) thought the probability was too high to be ignored. Smart angels agreed that the probability was negligible but asserted that it could not be claimed to be zero. And if it wasn't zero, to ignore it proved one to be a devil. Lovable angels argued that a little nonsense was a small price to pay for a safe code of practice for animal viruses. There were also naive lovable angels who were properly concerned about the spread of plasmid-borne drug resistance in bacteria. They wanted to ban the synthesis of bugs resistant to new combinations of antibiotics by gene splicing. When devils pointed out that to solve a problem the first step was to properly diagnose it, naive lovable

angels comforted themselves with the thought that a ban could do no harm, and it might even help just a bit.

Such a confusion of issues, such a mixture of motives, plus a widespread lack of awareness of population biology and ecology among the protagonists, were necessary raw materials for a controversy. But to raise the emotional temperature and politicise the issue required another factor. Pollack's spark needed a torch and it has been suggested to me by many people that the torch was the deep concern amounting to guilt of many United States scientists with the misuse of scientific knowledge—a concern that was born out of Los Alamos and came to a head for biologists when defoliants were used in an unpopular war. Lear's book offers support for this view at many points and a Roy Curtiss III, who emerges as its hero, is a good exemplar of this factor. He circulates one thousand copies of a sixteen-page signed testimony of self-criticism, admitting his innocent guilt and the frightening consequences of his work, pledging himself to be good in the future, and demanding much more severe constraints than were being mooted at the time. His stature in Lear's eyes is further enhanced by the revelation that he went to the library some years later and discovered that his work wasn't as frightening as he had thought. Thus he not only earns his role as a repository of the conscience of the scientific community but demonstrates his honesty and humility as well.

The bandwagon of public accountability came to a euphoric climax at Asilomar. A panel of assorted angels had been working hard on a code of practice which was accepted, after being modified a bit, because no one else had a better one. For the first time the scale of the hazards of genetic engineering were divined and graded and the revelations confirmed by a show of hands (allowing lesser mortals to publicly affirm their concern). For the first time the public could see that scientists believed that the theoretical hazards were more dangerous than real ones like, say, cholera or typhoid fever.

Before Asilomar public displays of concern could be indulged in at no cost. After Asilomar the bill was presented in the form of a blanket of controls out of all proportion to any reasonable assessment of hazards. Scientists handling totally harmless organisms found themselves embraced by a code of practice which assumed that they were dangerous pathogens. With minds thus concentrated there was growing pressure against the enshrinement of the code in legislation. Some people just went ahead and

ignored it. To Lear this lobbying and cheating proves his thesis that there are enemies of the people who will use any means fair or foul in the pursuit of unbridled scientific licence even to the point of danger to others. He has found his devils and in his enthusiasm to expose them his objectivity fails him. He even discovers a convention among scientists, which is flouted by his devils of course, but also by himself when it suits his purpose. This is the convention, new to me, that it is improper to cite unpublished work.

I should like to be able to record that those scientists who were collectively most influential in setting in motion the so-called debate on the hazards of genetic manipulation had, after due reflection, collectively used their influence to counterbalance the effect of their earlier statements. They have not done so and consequently Lear's thesis is bound to retain some credibility.

This book occasionally alludes to events abroad. The British Parliament reacted with alacrity to the 1974 petition and the Working Party it established completed its work with similar speed. We get a pat on the back for this but I fear that what it

demonstrates is the action of reflexes trained not to be caught with pants down in an unusually conservative society. Unfortunately, the same reflexes make any relaxation of the code of practice here much more difficult than in the more open and adventurous United States. Not yet for us the return to reason represented by the recent statement of the Director of the US National Institutes of Health that the burden of proof is shifting towards those who would restrict recombinant DNA research.

This episode should help to educate the public about science and scientists. The realisation that eminence does not necessarily equal wisdom, that expertise in one area can equal ignorance in another, that scientists are no more objective than anyone else, is healthy enough. The conclusion I draw from this is that on matters of policy we should base our decisions on the quality of arguments and not upon the status of those who expound them, as we have learned to do outside science. If this precept had been followed, Lear would have no story to tell. □

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Enigmas of filterable, invisible entities

David Baltimore

An Introduction to the History of Virology. By A. P. Waterson and Lise Wilkinson. Pp. 237. (Cambridge University Press: Cambridge and London, UK, 1978.) £12.

TODAY virology is a well defined discipline: it is the study of infective, intracellular parasites that lack the capability to synthesise their own proteins. This definition distinguishes viruses from other microbes and encompasses entities with sufficient similarities to provide the unity needed for defining a field of biology. But even up to the 1930s the essential nature of viruses was so obscure that the basic unity of their life-style was not apparent. All that was known about viruses before the advent of molecular biology was that they were small enough to pass through filters that trapped bacteria, they were invisible in the light microscope and they would not grow in the absence of living cells. And, of course, they caused a range of nasty diseases in humans,

other animals and plants; finding out more about them was therefore imperative.

The bacterial viruses, a group of viruses that were generally thought to be curiosities, generated the clues that brought the modern understanding of viruses. They provided the research materials from which much of molecular biology developed; and once the nature of genes and how they function was known, viruses became understandable creatures. The history of virology is thus a history of ideas as well as a history of discoveries. At first the recognition that certain diseases were caused by tiny, intracellular parasites was a triumph but the later realisation that these agents share the property of being stripped down to the bare essentials of replication was needed to provide a conceptual unity to virology.

There are so many viruses and the road to their understanding has been so complex that to write a history of virology would be an enormous undertaking. Waterson and Wilkinson were right to start with *An Introduction to the History of Virology*, in which they could define the outlines of the history. They did this with a strong emphasis on the conceptual problems.

The early part of their short book takes us back to late nineteenth century when Ivanovski and Beijerinck independently realised that what we now call tobacco

mosaic virus was so very much smaller than a bacterium that it ought to be considered a separate form of life. One must stand in awe of the pioneers of virology who, working with crude filters and imprecise assays, were able to bring to light a new aspect of the living world. In Beijerinck's earliest publications he originated the modern concept of a virus; the remarkable insight implied by his ability to make such a conceptual leap stands as one of the great intellectual achievements of biology. But, living up to their title, the authors only give us a passing taste of Beijerinck's work. Even a brief discussion of the origins of virology should tell us more about how Beijerinck derived his conclusions.

Another recent short book, Sally Smith Hughes' *The Virus: A History of the Concept* (Heinemann: London, 1977) provides a deeper look at Beijerinck's work. Hughes points to a remarkably simple experiment as the source of Beijerinck's concepts; he put infected plant sap on an agar surface and showed that the infective principle would diffuse through the agar. From this experiment he concluded that the virus is "dissolved and not corpuscular" leading to his concept of tobacco mosaic disease as a "contagium vivum fluidum". Beijerinck saw viruses in chemical terms, possibly as a partial result of his friendship with van't Hoff. This may have been the genesis of his pronouncement in 1898 that:

"... it is to some extent an explanation to consider that the contagium in order to reproduce itself must be incorporated into the living protoplasm of the cell into whose multiplication it is so to speak passively included."

After describing how Loeffler and Frosch opened animal virology with their discovery of the agent of foot and mouth disease virus, Waterson and Wilkinson take us through the discovery of a number of other animal viruses. They follow with discussions of vaccines and tissue culture. They emphasise the difficulty of understanding the nature of viruses without an appreciation of the molecular events underlying genetic phenomena and they show how bacterial viruses provided crucial experimental material for the development of molecular biology. In turn, they show how the development of molecular biology led to an understanding of the nature of viruses. After chapters on plant and insect viruses and on "eruptive fevers" they turn to tumour viruses, a subject that cries out for a more extensive history documenting the shifts of belief about the role of viruses in experimental and human cancer.

For someone like me, who entered virology in 1960 when the essential nature of viruses as intracellular parasites was commonly accepted, it is fascinating to see how the early virologists wrestled

with the enigmas of filterable, invisible entities that would not grow in the absence of living cells. Although this book is often a sketchy introduction and sometimes becomes a catalogue of events, it is a very valuable probe into a central aspect of the history of biology and medicine. Among its useful aspects is a collection of short biographies of

many of the important contributors to unravelling the puzzles presented by viral diseases. It also contains a list of general references and a useful bibliography of the original early literature. □

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Plant hormones

Phytohormones and Related Compounds: A Comprehensive Treatise. Vol 1: The Biochemistry of Phytohormones and Related Compounds. Edited by D. S. Letham, P. B. Goodwin and T. J. V. Higgins. Pp.641. (Elsevier/North-Holland/Biomedical: Amsterdam, Oxford and New York, 1978.) \$99.50; Dfl.224.

To be invited to review a book of this nature is a mixed blessing. It is, of course, a privilege to own a copy of the book; on the other hand, wading through 640 worthy but inexpressibly turgid pages in order to pronounce on its value to a prospective reader would be a thoroughly dismal task. I have not, therefore, read this book; I have sampled it. As it is questionable that books like this are ever meant to be read, then my approach is probably representative of any likely customer.

The book is sub-titled *A Comprehensive Treatise*, and this first volume undoubtedly lives up to that claim, although it does seem to be rather out of date. In a survey of the substantial reference lists given at the end of each chapter, I only encountered a handful of 1977 references, and these were principally those of the authors. Even 1976 seemed to be a rather rare date, suggesting that most of the preparation of the book was completed in mid-1976, with perhaps some special references added at the proof stage. Consequently, those looking for up-to-date reviews would be better advised to use *Annual Reviews*, or one of the other periodicals. As a solid compilation of the factual information relating to plant hormones, on the other hand, this book can have no equal.

The first chapter—which I did read—is a thought-provoking, partly historical, account of some of the more provocative general questions concerning plant hormones and their study. It is the only part of the book in which the conceptual content matches the factual content, and is thereby especially welcome. The density of fact, and its repetitive corroboration, is so high in the rest of the book as to make it difficult to digest. Apart from the first

chapter, one would be unlikely to open this book unless one had a specific technical point in mind. On the other hand, it would be invaluable for that purpose.

This first part of the treatise covers the biochemistry of hormones, and does so in the traditional manner, by considering each group of hormones separately. Chapters 2–6 cover auxins, gibberellins, cytokinins, ethylene and abscisic acid in a more or less uniform format, dealing with isolation, identification, chemistry, assays, structure-activity relationships, biosynthesis, metabolism, transport, and the effects of various stimuli on hormone levels. These chapters seem to me to be excellent contributions, and it is extremely valuable to have all this information in one source. Following these chapters is an extremely useful compilation of data on naturally occurring plant growth regulators other than the principal hormones.

The remaining chapters (over 200 pages) are concerned with the effects of hormones on cell structure and metabolism in general, and on DNA replication, transcription, translation, and on post-translational control points. I found the chapter on ultrastructural effects of the hormones particularly rewarding, as it emphasises the syndromic nature of hormone action in higher plants, and illustrates the naivety of assuming that cell-free studies will tell all. These chapters are all written by Drs T. J. V. Higgins and J. V. Jacobsen and represent a considerable, and as far as I could tell, highly critical, *tour-de-force*.

My impression, therefore, is of an excellent, though very viscous, treatise which should find a place on every plant physiologist's shelf. My absolute certainty that it will not do so, however, comes from the horrendous price. At \$99.50, or £50, this book—and remember it is yet only half a book—will not only be out of the reach of individual purchasers, it will also be too expensive for most college or university libraries. What purpose can there be in putting together a book which will only be available to a select few?

H. Smith

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Biological effects of ultraviolet radiation

UV-A: Biological Effects of Ultraviolet Radiation, with Emphasis on Human Responses to Longwave Ultraviolet. By J. A. Parrish, R. A. Anderson, F. Urbach and D. Pitts. Pp.262. (Plenum: New York and London, 1978.) £15.75.

It has long been known that sunlight has both beneficial and deleterious effects on man. Many of these effects are caused by the ultraviolet region of the solar spectrum, which has somewhat arbitrarily been divided into three sections—UV-C (wavelength 200–290 nm), UV-B (290–320 nm) and UV-A (320–400 nm). The biological responses of cells, of skin and of the eye to UV-C and UV-B have been well studied. On the other hand relatively little is known about the responses to UV-A, because on a dose-for-dose basis it is much less potent than the lower wavelength regions of the spectrum. The authors of *UV-A* stress that it is nevertheless of growing importance to assess the biological effects of UV-A.

Although it is of relatively low potency, the incidence of UV-A reaching the Earth from the Sun is very much higher than that of UV-B and UV-C. Also, UV-A is finding increasing use both in industry and in medicine, in the latter case particularly in conjunction with photosensitising drugs. Three problems in studying responses to UV-A crop up in almost every chapter: the inadequate dosimetry in most cases; the difficulty in obtaining sources of sufficiently high intensity; and the possibility that the effects observed may in fact be due to contamination of the UV-A with trace amounts of the much more potent UV-B. The authors have therefore wisely not confined themselves solely to UV-A but in each chapter they have discussed effects of ultraviolet radiation of different wavelengths and used this as a background against which to consider the (usually rather scanty) work with UV-A.

The book is very comprehensive in the range of subjects covered, commencing with light sources, filters and detectors, and proceeding with optical properties of the eye and skin—the parts of the body most affected by ultraviolet light. There follow chapters on the biological effects of ultraviolet light at various levels, starting with molecular studies on cells, and continuing with effects on the skin (erythema, tanning, skin ageing and skin cancer) and the eye (keratitis and cataracts). The book concludes with a

chapter on the uses of UV-A involving human exposure, and one on safety and protection. The former is chiefly concerned with photochemotherapy using psoralens and UV-A (PUVA) for the treatment of various skin ailments such as psoriasis and vitiligo. PUVA treatment is one of the few instances in which it is quite certain that the biological response is indeed mediated by UV-A rather than by shorter wavelength light. A balanced account is presented of the undoubted benefits of this therapeutic regime and the risk against which this must be offset, namely that in the long term the therapy itself may induce skin cancer.

A most impressive feature of this book is the way in which each topic is introduced with a succinct and lucid description of the system under study, so that the non-specialist reader is provided with the necessary basic information to understand what is subsequently discussed. This applies to

aspects of physics (for example, excited states), biochemistry (DNA repair), physiology (structure of the skin and eye) and medicine (description of psoriasis). The authors have therefore been remarkably successful in producing a book which should be comprehensible to anyone interested in the subject matter, whether he be physicist, biologist or clinician. As so little seems to be really known about the biological effects of UV-A at present, it is questionable whether the subject merits a whole book. On the other hand it contains much useful information on related topics. It will therefore be a handy reference book for any photobiologist and it should certainly be compulsory reading for anyone working with UV-A.

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Experimental phycology

Physiological and Biochemical Methods. Handbook of Phycological Methods. Edited by J. A. Hellebust and J. S. Craigie. Pp. 512. (Cambridge University Press: London, New York and Melbourne, 1978.) £18.

ALGAE serve as experimental tools for physiologists, biochemists, molecular biologists, organic chemists and so on, but the experimental techniques which these workers use are scattered throughout the literature. In 1967 the Phycological Society of America had the good sense to promote the publication of a source book on methods used in experimental phycology, written by phycologists. This one book never appeared; instead four were eventually commissioned, and although progress in getting these off the ground was slow initially (the first, on culture methods and growth measurements, appeared in 1973), the second, on physiological and biochemical methods, has been worth waiting for.

This is a "how to do it" book which should find a place on the shelves of any experimental phycologist. Not that I think it will remain on the shelves for long—it will be in constant use by graduate students, teachers, and others interested in algae. Its great advantage is that within the one cover there is such a mine of information. There are 46 chapters, by 46 authors (not necessarily one each), arranged in

seven major sections: isolation of organelles and membranes (how to isolate chloroplasts, microbodies, membranes, and so on); analysis of chemical constituents (pigments, protein, glycolipids and fatty acids, β -1,3 glucans, alginic acid, ATP, and so on); enzyme assays (nitrate reductase, ribulose 1,5-bisphosphate carboxylase, aldolases, and so on); measurement of physiological and biochemical processes (uptake of organic substrates, photosynthetic rates, nitrogenase activity, protein synthesis, and so on); nutrient uptake (nitrate, phosphate, and so on); ion content and transport; and use of inhibitors (a most useful chapter).

Each methods chapter is factual, with the sources of algal material (most workers on micro-algae still seem to use strains of *Chlamydomonas*, *Chlorella* or *Euglena*), equipment and chemicals (sometimes even the catalogue numbers) clearly given. There is usually a short discussion and some key references (few later than 1975). There is a useful appendix to the book which lists the addresses of many chemical and equipment suppliers and the suppliers of algal cultures (a notable exception is the American Type Culture Collection of Bacteria, where the blue-green algae are classified as cyanobacteria!)

A possible problem with such a book is trying to ensure that most of the important techniques are included and yet the price is not exorbitant. The book has most of the important ones but there are surprising omissions. One example: the key NH_4^+ -assimilating enzymes, glutamine synthetase and glutamic acid dehydrogenase, are not

considered, yet there is room for a chapter on threonine deaminase (an interesting chapter on a rather exotic enzyme). However, the overall balance is good with the more unusual features of algae as plants, for example, the production of alginic acid and fucoidan being adequately dealt with.

The only disappointing part of the book is the plates which are poor; the paper is also thin, but there

is a good stout cover and an adequate index. At £18 for over 500 pages packed with useful information I don't grudge a penny. I recommend it strongly to all with an interest in experimental phycology.

W. D. P. Stewart

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Neurochemistry and behaviour

The Chemistry of Human Behaviour. By H. L. Meltzer. Pp. 261. (Nelson-Hall: Chicago, 1978.) \$17.95.

BELIEF in the chemical governance or in the chemical signatures of behaviour has some centuries of history but little current exposition in popular writing. This book, which attempts "a progress report for the informed layman" on "knowledge of brain chemistry and how this might relate to human behaviour" thus had a welcome objective, but neither the expressed objective nor the book's title should be taken literally. Their embodiment is a collection of specialist essays in neuroscience, with a preponderance of neurochemical studies in experimental animals.

The subjects covered are for the most part already well collated or reviewed elsewhere and the text bears only occasional evidence of orientation to the informed layman. Indeed some parts will puzzle the informed chemist: arbitrary variations in bond-length in structural formulae, and giving for glutamine the structure of asparagine. The book's merits lie in giving a largely correct account of several research themes of the past 10 or 20 years, in which chemical methods have been applied in understanding the structure or working of the brain and its influence on behaviour. These themes include malnutrition and brain development; inborn chemical abnormalities; the ion exchanges of nerve impulses; neurotransmitters; the actions of drugs including amphetamines, opiates, lysergic acid diethylamide, and steroid hormones; and chemical correlates of learning and of mental illness. The book's index and Figures are satisfactory, and it carries a 5-page list of books and reviews suggested for further reading. The penultimate chapter on ethical considerations is an excellent and timely exposition of the reasonable researcher's approach to experimentation with animals and human subjects. The chapters on flow of information and on the physical struc-

ture of the brain, though non-chemical, are also apposite.

A book designated to give informed laymen an appreciation of a developing technical subject presents its own specific problems which have been met only partially by Dr Meltzer. His writing in general is crisp, factual and without involved sentences. Some chapters, however, are constructed to attract attention to an extent which, by the chapter's end, is disappointed. Some carry an introductory paragraph only tenuously related to the chapter's main subject; others imply by their title more than current knowledge justifies. Thus "The Chemical Organisation of the Brain" might lead the reasonable layman to expect an exposition of how and why the numerous chemical structures described and illustrated are organised, or organise other components, to yield a brain rather than another organ of the body. Here he will be disappointed and will learn only on the last of the 20 pages involved, that "This has been a brief survey of chemical localisation in the brain . . . intended as an introduction to the diversity of chemical function. . . ."

The most successful expositions of technical knowledge of the brain for general readers have, recently, come from neurophysiology, neuroanatomy or psychology. It is significant that the authors concerned with those expositions have not attempted to survey a large proportion of the current research in their subject: rather, they have chosen an intellectually intriguing theme central to their own interests and have been highly selective in the information presented to expound their viewpoints. Their pictures of the brain have included little if any neurochemistry at many points where neurochemistry would have been relevant. Comparably intriguing expositions from a neurochemical viewpoint have yet to be written: despite Dr Meltzer's book, the challenge remains open.

Henry McIlwain

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Investigating Animal Abundance

Capture-recapture for biologists

Michael Begon

By stressing the importance of biological context and the commonsense basis of the methods, this book aims to give biologists, at all levels, the confidence to use capture-recapture models for the assessment of animal population size, and to interpret the results they obtain sensibly.

The mathematical details of a variety of methods are treated in a clear and often novel way that requires little mathematical knowledge and there are useful sections on the actual techniques of capture and marking.

Paper £3.95

Publication March

An Introduction to the Interpretation of Quantal Responses in Biology

P. S. Hewlett and R. L. Plackett

This guide to the interpretation of quantal (all-or-none) responses to such agents as pesticides or drugs covers both probit and logit analysis, although the latter — which is mathematically the simpler — is given prominence. The authors deal in a distinctive manner with the theory of responses to mixtures of drugs, and discuss certain topics rarely touched on in other books: namely, dose-response curves for heterogeneous populations, the theory of monitoring insect populations for resistance, time and response, and the general nature of quantal responses.

Paper £3.95

Publication March

Investigating Chromosomes

Adrian F. Dyer

This book strikes a balance between the theoretical and the practical aspects of cytogenetics. Assuming little prior knowledge, the local theoretical exposition is matched at every stage by practical sections that allow the student to investigate or visualise each stage of chromosome activity as it is introduced. All the practical routines are variations on one basic technique and are applied to a wide variety of plant and animal material.

Paper £5 approx

Publication Summer



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Sedimentary environments

Sedimentary Environments and Facies. Edited by H. G. Reading. Pp. 557. (Blackwell Scientific: Oxford, UK, 1978.) Hardback £22.50; paperback £13.

THIS book describes modern sedimentary environments and their ancient analogues. For each major facies the editor has arranged for a chapter to be written by an internationally recognised authority on that particular topic. The book commences with an introductory chapter and a chapter on facies by the editor. These are followed by chapters on alluvial, lacustrine and desert deposits by Collinson. Elliott continues with chapters on deltas and clastic shorelines, and these are followed by a chapter by Till on arid shorelines and evaporites. Johnson has contributed a chapter on shallow siliclastic seas (*sic*) and this is followed by one from Sellwood on shallow-water carbonates.

Moving into deeper water Jenkyns and Rupke cover pelagic environments and deep clastic seas respectively. Edwards concludes the spectrum of environments with a contribution on glacial deposits. For a finale Reading and Mitchell have written a chapter relating facies to plate tectonics and the book closes with an epilogue from the editor, a bibliography and a subject index.

As the preceding resumé shows, this work is a veritable *tour de force*. It is well researched, well written and comprehensive, both in the range of environments described and in the global coverage of supporting case-histories. The illustrations all appear to have been redrawn from their original sources so that the book has a pleasing uniform appearance (though this review copy had several grey pages due to an apparent shortage of printers ink).

The emphasis of most of the authors is on the relationship between sedimentary processes and the macroscopic features of their deposits. Thus, the major theme of the book is concerned with the study of lithological sequences and their contained sedimentary structures and fossils.

The emphasis is on macrofacies rather than microfacies. It would have been interesting to have had contributions from this distinguished team of authors on the relationship between sedimentary processes, and the texture and petrophysics of their deposits. This would have widened the appeal of the book for many industrial geologists concerned with the relationship between facies, porosity and permeability. This absence of attention to microfacies is particularly apparent in the

sections on carbonates, which contain not a single microphotograph.

Similarly it would have been useful to read how the authors see facies analysis in relation to other branches of geology. One of the most stimulating chapters is the one which shows how facies relate to tectonics. It would be interesting to know more about the relationship of facies to stratigraphy (both conventional and seismic), to microfacies and petrophysics (as already mentioned), and to the ways in which facies analysis is applied in the exploration for coal, oil, gas, water and sedimentary ores. This deficiency is particularly apparent when subsurface case-histories are described. It is not sufficiently stressed that these have been interpreted largely from microfacies and wireline logs, as cores are

seldom available for the detailed study of their macroscopic features. Only two figures in the book show the use of wireline logs in subsurface facies analysis. No doubt space was the reason why these topics were omitted, and indeed the editor is to be congratulated on the way so much valuable material has been compressed into 550 pages.

There is no doubt that this scholarly book will be invaluable as a text for senior undergraduate and postgraduate students aiming for an academic career. It will also be an essential source book for all geologists concerned with sedimentary rocks.

R. C. Selley

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Meteorite reference work

The Nature and Origin of Meteorites. By D. W. Sears. Pp. 187. (Adam Hilger: Bristol, UK, 1978.) £13.50.

THIS is an excellent reference work on meteorites, being comprehensive in scope, yet succinct. The book is divided into six chapters. The first is a historical introduction which makes interesting reading and helps put the succeeding chapters in their proper context. I was glad that "the tortuous and sometimes confusing route by which modern classification schemes have emerged" was dealt with briefly. This is a useful chapter because it indicates how closely advances in physics, chemistry and the Earth sciences have been bound to meteorite research; and this is still true.

Chapter 2 deals with atmospheric flight and impact phenomena, including tektites, which are probably produced in rare, high energy events by fusion or vaporisation of terrestrial materials. Various casehistories of meteorite falls are given. Statistics indicate that, over the Earth as a whole, two or three meteorites arrive per day, yet samples from only about six observed falls per year actually reach the scientist. Next comes 'Classification, mineralogy and petrology'. Here the author, in my opinion rightly, concentrates on the major groups of meteorites, key references to the others being given. The scope of the chapter is, however, wider than the title implies, for estimates of the sizes of meteorite parent-bodies are included. The fourth chapter is on chemical composition and covers the observational data and their theoretical modelling and interpretation. In view of the

sometimes bitter antagonism among those engaged in the field, Sears has done well to present the different cases lucidly, with fair indication of their strengths and pit-falls. Then comes 'Physical properties and processes', including age and isotopic studies. Unfortunately there is no reference to the samarium-neodymium results which have recently made a strong impact on our understanding of the history of terrestrial rocks.

The last chapter is a synthesis of our ideas on the age and origin of meteorites. Because of its extreme brevity, uninitiated readers will have to refer to the preceding text, but most of the necessary cross-references are there.

The book is well produced and contains few errors; on p121 the seismic velocity in sandstone is given as 3,000 to 4,000 km per second (it should be m per second), and I am sure that I would enjoy studying a 'cosmogenic tract' (p148). Table 4.1, on meteorite compositions, contains numerous errors and is useless for reference. Both author and publisher are to be congratulated in producing the book so quickly that references up to early 1978 are included, but I wish that titles had not been omitted.

From the points of view of price, accuracy and being up to date, I can recommend this as a good reference text. However, in spite of the statement on the dust-cover, it will be beyond the comprehension of most amateur astronomers and geologists, and is probably more advanced than is necessary for undergraduates. It will be most useful for research students and academics; they will be unable to do better for the price.

Robert Hutchison

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Recent scientific and technical books

Mathematics

- ANDREWS, George E. (edited by). Percy Alexander MacMahon Collected Papers. Vol. 1: Combinatorics. (Mathematicians in Our Time.) Pp.xxvii+1438. ISBN-0-262-13121-8. (Cambridge, Mass. and London: The MIT Press, 1978.) £75.
- BRIDGES, D. S. Constructive Functional Analysis. (Research Notes in Mathematics, No. 28.) Pp.203. ISBN-0-283-08418-6. (London, San Francisco and Melbourne: Pitman Publishing, Ltd., 1979.) £7.50.
- DAY, A. Colin. Compatible Fortran. Pp.vii+107. ISBN-0-521-22027-0. (Cambridge, London and New York: Cambridge University Press, 1978.) £5.95.
- GALLAIRE, Hervé, and MINKER, Jack (edited by). Logic and Data Bases. Pp.viii+458. ISBN-0-306-40060-X. (New York and London: Plenum Press, 1978.) £18.58.
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- KEMENY, John G., and SNELL, J. Laurie. Mathematical Models in the Social Sciences. Pp.vii+145. ISBN-0-262-61030-2. (Cambridge, Mass. and London: The MIT Press, 1978.) Hard \$10.95; Paper \$5.95.
- KLINE, Morris (with introductions by). Mathematics: An Introduction to Its Spirit and Use. (Readings from *Scientific American*.) Pp. 249. ISBN-0-7167-0369-6. (San Francisco and Reading: W. H. Freeman and Company, 1979.) Hardcover £8.90; Softcover £4.70.
- ROTA, Gian-Carlo (edited by). Studies in Foundations and Combinations. (Advances in Mathematics: Supplementary Studies, Vol. 2.) Pp.ix+265. ISBN-0-12-599101-0. (New York and London: Academic Press, a Subsidiary of Harcourt Brace Jovanovich, Publishers, 1978.) £18.85.
- ROTA, Gian-Carlo (edited by). Studies in Probability and Ergodic Theory. (Advances in Mathematics: Supplementary Studies, Vol. 2.) Pp.xiii+293. ISBN-0-12-599102-9. (New York and London: Academic Press, a Subsidiary of Harcourt Brace Jovanovich, Publishers, 1978.) £32; £20.80.
- STEEN, Lynn Arthur (edited by). Mathematics Today: Twelve Informal Essays. Pp.viii+367. ISBN-3-540-90305-4. (Berlin and New York: Springer-Verlag, 1978.) DM 27; £14.90.
- SZIDARIVSKY, Ferenc, and YAKOWITZ, Sidney. Principles and Procedures of Numerical Analysis. (Mathematical Concepts and Methods in Science and Engineering, Vol. 14.) Pp.xiii+331. ISBN-0-306-40087-1. (New York and London: Plenum Press, 1978.) £15.43.
- VAN OYSTAEYEN, F. (edited by). Ring Theory. (Proceedings of the 1977 Antwerp Conference.) (Lecture Notes in Pure and Applied Mathematics, Vol. 40.) Pp.vii+207. ISBN-0-8247-6814-0. (New York and Basel: Marcel Dekker, Inc., 1978.) Sw. fr. 50.
- VAN ROOIJ, A. C. M. Non-Archimedean Functional Analysis. (Pure and Applied Mathematics: a Series of Monographs and Textbooks, Vol. 51.) Pp.x+404. ISBN-0-8247-6556-7. (New York and Basel: Marcel Dekker, Inc., 1978.) Sw. fr. 64.
- WANG, Peter C. C. (edited by). Graphical Representation of Multivariate Data. (Proceedings of the Symposium, Naval Postgraduate School, Monterey, California, February 24, 1978.) Pp.ix+278. ISBN-0-12-734750-X. (New York and London: Academic Press, a Subsidiary of Harcourt Brace Jovanovich, Publishers 1978.) £15; £9.75.
- WETHERILL, G. Barrie. Sequential Methods in Statistics. (Monographs on Applied Probability and Statistics.) Pp.x+232. ISBN-0-412-21810-0. (London: Chapman and Hall, 1979. Distributed in the USA by Halsted Press, a Division of John Wiley and Sons, Inc., New York.) £4.50.
- WITTGENSTEIN, Ludwig. Remarks on the Foundations of Mathematics. Edited by G. H. von Wright, R. Rhees and G. E. M. Anscombe. Translated by G. E. M. Anscombe. Revised edition. Pp.444. ISBN-0-262-23080-1. (Cambridge Mass.: The MIT Press; Oxford; Basil Blackwell, 1978.) £27.50.

Physics

- ALEFELD, G., and VOLKL, J. (edited by). Hydrogen in Metals II: Application-Oriented Properties. With contributions by G. Alefeld, et al. (Topics in Applied Physics, Vol. 29.) Pp.xvii+387. ISBN-3-540-08883-0. (Berlin and New York: Springer-Verlag, 1978.) DM 86; \$47.30.
- ARECCHI, F. T., BONIFACIO, R. and SCULLY, M. O. (edited by). Coherence in Spectroscopy and Modern Physics. (NATO Advanced Study Institutes Series, Series B: Physics, Vol. 37.) Pp.viii+1402. ISBN-0-306-40050-2. (New York and London: Plenum Press, 1978. Published in co-operation with NATO Scientific Affairs Division.) £24.88.
- BATEMAN, Glenn. MHD Instabilities. Pp.ix+263. ISBN-0-262-02131-5. (Cambridge, Mass. and London: The MIT Press, 1978.) £22.50.
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- BRUNDLE, C. R., and BAKER, A. D. (edited by). Electron Spectroscopy: Theory, Techniques and Applications, Vol. 2. Pp.xi+287. ISBN-0-12-137802-0. (London and New York: Academic Press, a Subsidiary of Harcourt Brace Jovanovich, Publishers, 1978.) £17.50; \$35.25.
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obituary

Alexander Wetmore

ALEXANDER WETMORE, who died in Maryland on 7 December 1978 at the age of 92, was for over 50 years one of the leading figures in world ornithology. Born in Wisconsin on 18 June 1886, he lived all his life in the United States, devoting his professional life to two organisations, first the Biological Survey of the Department of Agriculture (1910–1924) and from 1924 the Smithsonian Institution. From 1945 to 1952 he served as Secretary of the Smithsonian Institution, and he continued to work there after his retirement until a short time before his death.

A good many living and recent ornithologists have had new subspecies, and even the occasional new species of bird named after them; but few have had the distinction of giving their name to a new genus, such as *Wetmore-thraupis*, a new tanager of uncertain affinities discovered in northern Peru in 1963 and named by Lowery & O'Neill in Wetmore's honour 'in recognition of his many outstanding contributions to ornithology, ranging from the classification and systematics of both recent and fossil birds to such diverse facets of the subject as bird migration and pterylosis.' Indeed Wetmore's name has become a household word among ornithologists, for his classification of the birds of the world, forming the basis for the well known 'Wetmore order', has, with modifications necessitated by new knowledge, become the generally accepted arrangement, as adopted in Peters' *Checklist of Birds of the World* and other authoritative compilations.

Dr Wetmore's eminence as a research ornithologist was based on an extraordinary capacity for work, meticulous accuracy, and a very wide range of interests. When he was Secretary of the Smithsonian he customarily arrived at his laboratory at about 6 a.m. and worked steadily on his scientific projects for three or four hours before devoting a full day's work to his duties as Secretary. Up to his late 80s he continued to make regular field trips to Panama, collecting specimens and gathering information for his 4-volume *Birds of the Republic of Panama*, the last volume of which was left unfinished at his death. These qualities, applied to avian palaeontology at a time when the subject was in a state of comparative neglect, quickly made him a leading authority, his con-

tributions providing almost all that was known of the birds from the extensive Oligocene deposits of western North America. Since 1914 he described as new 189 species and subspecies of birds, a record that must be unequalled in the 20th century.

Dr Wetmore was, in a sense, an old-fashioned ornithologist, museum-based, his field work centred round collecting—one who provides the facts that others use. He did not originate new theories, seeming distrustful of the sweeping generalisation, as is often the case with those who are very familiar with the facts and see, more clearly than most, the exceptions and difficulties that need to be taken into account. Advanced though it is in the accumulation of basic facts, ornithology still needs workers of his type; they provide the infrastructure that supports the more exciting new developments.

Alexander Wetmore was a tall man of quietly distinguished presence and great natural modesty, who treated the youngest aspiring ornithologist and the older established with equal courtesy. I never heard any of his American colleagues or friends speak of him other than with respect and affection. He will be remembered by the many British ornithologists who met him at the International Ornithological Congress at Oxford in 1966, still in full vigour and only regretting that he was becoming less efficient in the field as his hearing was beginning to fail.

D. W. Snow

Sidnie Manton

DR SIDNIE MANTON, FLS, FRS (Mrs J. P. Harding), died peacefully in hospital on 2 January 1979 aged 76. To students of zoology at the end of the last war she was already a legend.

In her first 20 years of professional life she had jointly opened the era of functional morphology with Graham Cannon in their paper on the feeding mechanism of *Hemimysis*; journeyed to Tasmania to study the endemic *Anaspides*; joined the expedition to the Great Barrier Reef and published on black corals; published on the embryology of *Nebalia*; sought-out *Peripatus* in Africa, and published on their feeding, digestion, excretion, sperm trans-

fer, embryology and life-history. A graduate and fellow of Girton, she became college director of studies in natural science, university demonstrator in comparative anatomy and later joined the staff at King's College, London. Her *Manual of Practical Vertebrate Morphology* (1930, with J. T. Saunders) was, and still is (4th edition, 1969, with M. E. Varley) the most respected and reliable guide to vertebrate dissection.

Her election to the Royal Society in 1948 crowned a distinguished career and came as no surprise. No one could have guessed that she was poised to make an even more important contribution to scholarship in the next 25 years. A gap in publication from 1938 to 1945 represents the period her young family and a heavy teaching load at Kings, Birkbeck, Queen Mary and University Colleges were making big demands on her time, but it was then she conceived and began one of the most exciting and ambitious research projects in the history of zoology. The first part of this already classic series on the evolution of arthropod locomotory mechanisms was published in the *Journal of the Linnean Society* (Zoology) in 1950 and the final part (11) appeared in 1973. The series covered 1,000 pages and included almost as many superbly executed drawings gathered into 250 compound text-figures. Functional explanations were given of many arthropod characters previously regarded as curious indications of ancestry.

She was continually in demand here and abroad, to talk about her work. At one Linnean meeting she brought along working models of arthropod legs she had made to help her own understanding; that of the spider was as tall as she, and incorporated the arcuate sclerite, a marvellous engineering device to provide the acute flexure of the femur-patella joint. The nicest device she discovered was surely the rock of the arthropod coxae, lining-up the dorsal hinge joints to translate the depression of proximal podomeres into effective extension of the legs. She was awarded the Linnean Gold Medal in 1963 before half the series was completed.

The locomotion work was certainly conceived and executed in the grand manner, but it was merely a part of

the greater scheme conceived much earlier, aimed at an understanding of arthropod phylogeny. She had extended and completed a draft of the late O. W. Tiegs on *The Evolution of the Arthropoda* and had discovered that much basic information was just not available. In 1960 she retired from her Readership at Kings and from then on enjoyed the hospitality of the British Museum (Natural History) as Honorary Research Associate and was also a Research Fellow of Queen Mary College. Freed from teaching she felt able to seek the information for herself, beginning with a major examination of the structure and function of the mandible which was published in a wonderfully illustrated paper of over 200 pages between parts 7 and 8 of the locomotion series. She was becoming increasingly impressed by the mutual exclusiveness of the engineering designs between and even within classes. She considered these designs to have evolved in response to different habits which persisted for long periods and that each lineage represented an independent attainment of the arthropod grade of organisation.

About this time she was cruelly afflicted by an arthritic condition, yet she had enjoyed lunch-time tennis on the BM court until she was well over 60. At the Myriapod Congress in Manchester in 1972 she was relatively mobile because of an ingenious mechanical aid for one leg designed and constructed by herself and husband. Looking down a microscope at an exhibit, she drew for me an accurate picture of the cataract which limited her field to a narrow jagged annulus. Cataracts were removed one by one and hip and wrist joints were extensively re-modelled. During this difficult period she wrote *The Arthropoda*, recently published by Oxford University Press, a masterly summary of her 50 years research into their habits, functional morphology and evolution, running to 527 pages and lavishly illustrated by 186 text figures. She corrected the proofs in hospital after one of her operations. During the last two years she had compiled four chapters of a new general text on arthropods, written and presented a paper to a symposium on the evolution of the major arthropod groups at Hull, contributed a review to a book on arthropod phylogeny and undergone a second pelvic joint operation. The strain was too great.

The arthropods were not the only animals in her life. Her home and garden housed a veritable menagerie of birds and mammals which she cared for each day. Over a period of 21 years she developed the long haired Colourpoint cats with such delightful tem-

peraments and exported kittens to all points of the globe. The history of her Mingchui cats is recounted in her book *Colourpoint, Longhair and Himalayan Cats* (1971). This work ran parallel to her arthropod studies and brought her world-wide acclaim from an entirely different public. All this while at the centre of a delightful household, cooking superb meals, a delightful person with a lively conversation and an understanding smile. She will be sorely missed by her husband, Dr J. P. Harding and her two children, Elizabeth and Martyn.

J. Gordon Blower

E. R. Creed

EDWIN ROBERT CREED died on 28 November 1978, aged 42, after an illness of three months. His parents were both physiologists, his father being a Fellow of New College, Oxford, also of Winchester. Robert was among that group of boys from Marlborough School who, from the 1920s onwards, became distinguished as biologists.

Like his father, he went to Trinity College, Oxford. After graduating in zoology he remained in the department to carry out research in ecological genetics. It gained for him the PhD Degree, also a Research Fellowship at New College. When this expired in 1970 under a time-limit, he became Lecturer, and later Senior Lecturer, in Zoology at University College, Cardiff, where he was in charge of genetics.

Unlike many scientists and scholars in general, Robert Creed did not find his period of National Service a disastrous waste of time. Among other things, it enabled him to develop his ability in engineering. Indeed, he retained what to some of us seemed an astonishing capacity to interchange and adjust the parts of old motor cars, of which he always had several. Moreover, for the rest of his life he took an active interest in the Territorial Army Volunteer Reserve.

His marriage to Elizabeth Hilton was a source of great happiness. He leaves two daughters, one aged 7 and the other 4 years. He liked to live in fairly large houses plentifully supplied with cats.

He was a good lecturer, but his outstanding educational achievement was as a demonstrator in practical classes. Here he excelled. He would take great care to arrange interesting exhibits and experiments. It was his habit to take away and work up the class results, and he would subsequently explain in what ways they had proved rewarding or unusual.

He was also a successful research worker. In order to investigate the ecological genetics of the butterfly *Maniola jurtina* he once camped with me on an uninhabited island in the Isles of Scilly, and he repeatedly joined our research group studying that insect in the south-west of England. He had a flair for such work, in which his observations were accurate and often revealing; and on these occasions his light-hearted energy was an asset and an encouragement.

His own researches were in particular directed to the polymorphism of the two-spot ladybeetle, *Adalia bipunctata*. This is typically scarlet with a black spot on each wing-cover; but two black forms with, respectively, four and six red spots are also known, all three types being controlled as multiple alleles. Creed showed that the black phases are industrial melanics, the only known instance of that condition in a species protected from predation by a powerfully unpleasant scent and taste: one practising, therefore, the aposematic way of life.

Creed and his colleagues also found that the black phases of *A. bipunctata* are favoured when pollution results from coal of high volatility, not of low volatility as in South Wales; nor does the presence of sulphur dioxide in the atmosphere encourage their spread, as in some moths. Moreover, the two black forms respectively survive better when the fuel is, or is not, subject strongly to caking. This work, described in his published articles, is a valuable contribution to our knowledge of adaptation to industrial conditions.

In spite of his youthful appearance, Robert Creed had a highly developed critical faculty and his comments when the draft of an article was submitted to him were of penetrating value. He was a Conservative in politics, original in research, devoted in friendship. His early death is a tragic loss of one who can ill be spared.

E. B. Ford

Lord Cranbrook

THE death of John David, 4th Earl of Cranbrook on 22 November 1978 deprives many natural history and scientific societies of a skilful adviser and an enthusiastic and knowledgeable colleague.

His multifarious activities in public service and in support of East Anglian naturalists' organisations have been acclaimed elsewhere (*The Times*, 24 November 1978) but it is for his capacities as a scientist of no mean distinction and as a staunch member and officer of national biological societies that we, on behalf of the

Linnean Society of London and of the Mammal Society, would wish him to be remembered. We have no doubt that other societies, such as the Zoological Society of London, would wholeheartedly support this tribute.

Lord Cranbrook's schooling as a naturalist with a world-wide field of interest began in the Far East in association with such pioneers as Kingdon Ward. Thereafter he was deeply committed to the furtherance of field biological studies in Great Britain.

For over 46 years he laboured on behalf of our oldest biological society, the Linnean Society of London, which has always acted as a fruitful meeting ground for amateurs (of which he was an outstanding example) and professionals. He served on its Council for 14 years and was Treasurer for 12 years. His astute business sense, combined with his robust common sense, proved invaluable to the Society during its special appeal for funds in 1969. As a result of this appeal, the Linnean Society was able to rehouse and adequately curate its unique collections of plants and animals on which Linneaus once worked; a service which was appreciated by scientists throughout the world.

Lord Cranbrook's enthusiasm, energy and eminence among his wide contacts put him in a unique position to foster progress. Nobody appealed to him in vain for advice and it was always given widely, humorously and forcibly.

Compared with the Linnean, the Mammal Society is a relative newcomer with no valuable collections to house and curate. Lord Cranbrook was a founder member in 1954 and, as its assiduous President, guided the Society in its formative years. It was rare for him to miss a council meeting and each year, when he presided over the Annual General Meeting, there was keen speculation as to whether he could cut ten minutes off his previous record for its speedy completion. Nevertheless, there was no skimping and his grasp of essentials, together with his succinct exposition, gave every point due consideration.

As a Trustee of the British Museum (Natural History) between 1963 and 1973, he again provided a valuable link between the naturalists at large and the more circumscribed specialists.

Lord Cranbrook was a valued speaker in the House of Lords: valued especially because of the reliability of his 'homework' and the clarity and force with which he dealt with complex and controversial matters. All naturalists must be grateful for his pilotage during recent years, when he faced increasing disability with great courage, of the Conservation of Wild Creatures and Wild Plants Act 1975.

He contributed numerous publications to scientific journals, of which the most outstanding was his study of a population of noctule bats which came to feed on crickets at a local rubbish dump. Capturing and marking them with the aid of mist-nets was a notable achievement at that time (1965, *Proc. zool. Soc. Lond.* **144**: 1-24).

Lord Cranbrook's cheerful presence and leadership will be sorely missed throughout the ranks of naturalists and biologists.

P. H. Greenwood
H. N. Southern

Jean Clark Dan

JEAN CLARK DAN died of asthma in Tateyama, Japan on 13 November 1978, at age 68. Born and reared in a staunchly Presbyterian family in Westfield, New Jersey, USA, she graduated from Wilson College (1932), and continued study under L. V. Heilbrunn at the University of Pennsylvania and in summer at the Marine Biological Laboratory, Woods Hole. On obtaining her PhD (1936) she married Katsuma Dan who had also received his PhD (1934) with Heilbrunn. They proceeded to Japan via Europe with a stay at the Stazione Zoologica of Naples. Eventually they settled in Tokyo but their early years included long periods at the Misaki Marine Biological Station of Tokyo University.

The Dans produced five children, whose upbringing overlapped World War II. Jean had to move away from home territory but adapted cheerfully, and despite many hardships the whole family managed to survive. Then she undertook multifarious enterprises to surmount postwar adversity: one negotiation utilized army garbage for village rehabilitation while preserving self-respect all around. And she doggedly struggled back to the scientific world.

In 1947 she revisited Woods Hole (to which she would often again return as to another home) where peacetime activities had been re-established. With help from the American Philosophical Society she took a phase contrast microscope back to Japan where, under the wing of Tokyo Metropolitan University, she set up at Misaki a research program on fertilization of marine invertebrates. Publication soon followed. The papers became increasingly important. Her first two 'Studies on the Acrosome' (1952, 1954) demonstrated how the tip of the spermatozoon changes under various conditions, and introduced the term

acrosome reaction. She was a pioneer in examining such material with the electron microscope. Some workers were slow to accept her findings but her mounting evidence, together with that of others, firmly established that the reaction does indeed occur. There followed many other important papers. She explored the sperm lysis problem. Much of her later observation and speculation centered around 'dynamic morphology' (precursors, triggers, etc.) of membranous and other parts of the region she came to call the *acrosomal complex*.

She won grants and fellowships from sources in both countries, received her Japanese PhD, was given the award (1958) of the Zoological Society of Japan, and found herself in international demand for lectures and conferences on spermatology and fertilisation. She was on the editorial board of *Development, Growth and Differentiation*.

Jean was appointed Lecturer (1959), then Professor (1973) at Ochanomizu University where she taught and supervised graduate students. Interested in that University's Tateyama Marine Biological Station from its inception, she became Director (1975), and planned to spend much of her time at Tateyama after retiring from the University (1976).

During the war Jean had come to speak fluent Japanese. As Japanese biologists returned to research and writing many sought her help with English usage. For years she devotedly helped transform their texts into English suitable for publication. She translated into English Osada's *Children of the A-Bomb* (1959, with R. Sieben-Morgen), and *Invertebrate Embryology* (1968, eds. M. Kumé and K. Dan). Moreover, she assisted in the English translation of publications of His Majesty, Emperor Hirohito.

The above barely suggests the warm vital Jean Dan. She was a strong swimmer, walked well, liked to gather wild mushrooms or buy a warm sweet-potato from a street vendor. She liked having a dog. Her children enjoyed a whole family of Siamese cats. In her laboratory would be a flower in a beaker, an attractive picture on the wall. She enthusiastically espoused causes as great as world peace, and with equal vigor would propel a specific student into opportunities for advanced study. Jean was seldom at a loss for words; also, her penchant for friendly listening to and advising others knew no barrier in age, sex or nationality, discipline or problem.

She will be missed as a scientist—and as a person.

Laura Hunter Colwin
Arthur L. Colwin